The effect of food storage conditions on the survival of *Campylobacter jejuni*

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Abbreviations

1D-PAGE = One dimensional polyacrylamide gel electrophoresis

2D-PAGE = Two dimensional polyacrylamide gel electrophoresis

A = adenine

ABCD = ACES buffered chemically defined medium

ABNC = active but non-culturable

ACES = N-2-acetamido-2-aminoethanesulphonic acid

ADP = adenosine diphosphate

AhpC = alkyl hydroperoxide reductase C subunit

AMP = adenosine monophosphate

AO = acridine orange

AODC = acridine orange direct count

APS = ammonium persulphate

ATP = adenosine triphosphate

bp = base pair

BSA = bovine serum albumin

C = cytosine

CCCP = carbonyl cyanide *m*-chlorophenylhydrazone

CFDA = 6-carboxyfluorescein diacetate

cfu = colony forming units

CFW = calcofluor white

CHAPS = (3-[(3-cholamidopropyl) dimethylammoni)-1-propane-sulphonate

cm = centimetre

CTC = 5-cyano-2,3-ditolyl tetrazolium chloride

d = days

Da = daltons

DAPI = diamidinophenylindole

DMSO = dimethylsulphoxide

DNA = deoxyribonucleic acid

DTT = dithiothreitol

DVC = direct viable count

EA = enhanced autoradiography

EDTA = ethylenediaminetetraacetic acid

ergs = ergs (unit of work done by a force of one dyne over a distance of 1 cm)

FBP = iron (II) sulphate (F), sodium metabisulphite (B), sodium pyruvate (P)

FS = forward scatter

g = grams/ gravitational force

G = guanine

GBS = Guillain-Barré syndrome

GFP = green fluorescent protein

h = hours

IEF = isoelectric focusing

INT = 2-(4-iodonitrophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride

IPG = immobilised pH gradient

kDa = kilodaltons

kV = kilovolts

LPS = lipopolysaccharide

M = molarity

mA = milliamps

MEM = minimal essential medium

μg = microgram

μm = micrometre

μM = micromolar

mg = milligrams

min = minutes

mM = millimolar

mol. wt = molecular weight

MPN = most-probable number

M_r = relative molecular weight

NADH = nicotinamide adenine dinucleotide (reduced)

NIC = Northern Ireland Chicken

NP-40 = nonidet P-40

OMP = outer membrane protein

PBP = penicillin binding protein

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PG = peptidoglycan

PHLS = Public Health Laboratory Service

pH = - log hydrogen ion concentration

PI = propidium iodide

pmf = protonmotive force

PMSF = phenylmethylsulphonyl fluoride

pppGpp = guanosine pentaphosphate

psi = per square inch (pressure)

revs min^{-1} = revolutions per minute

Rh123 = rhodamine 123

RNA = ribonucleic acid

secs = seconds

SDS = sodium dodecylsulphate

SOD = superoxide dismutase

SRM = silicone release material

SS = side scatter

T = thymidine

TCA = trichloroacetic acid/ tricarboxylic acid

TEMED = N, N, N, N', N'- tetramethylethylethylenediamine

TNACM = The National Advisory Committee for Microbiology

Tris = tris (hydroxyethyl) aminoethane

TSP = trisodium phosphate

UV = ultraviolet

VBNC = viable but non-culturable

v/v = volume per volume

W = watts

w/v = weight per volume

Abstract

Campylobacter jejuni is the predominant cause of gastro-enteritis in the developed world, accounting for 58, 000 diagnosed cases in the U.K in 1998. Infection primarily results from consumption of undercooked poultry and poor preparatory hygiene. *C. jejuni* is a poultry commensal, hence products are frequently contaminated within the processing plant, and subsequently at retail. Survival under conditions imposed by poultry processing and storage is an important factor in the capability of *C. jejuni* to produce infection.

C. jejuni cells are usually spiral, but can be converted to a spherical form (coccoid) under various conditions. A triphasic survival curve is exhibited upon exposure to cold-shock (4-20 °C), consisting of plateau, decline and non-plateable phases. Maximum survival was observed for stationary phase cells incubated at 4 °C under a microaerobic atmosphere. Loss of plating ability on Brucella-FBP medium occurred before coccoid transformation at all temperatures examined (37, 20 and 4 °C) indicating the formation of a non-plateable spiral state.

Entry into the non-plateable state correlated with an increase in calcofluor white (CFW) staining. When the population of CFW-stained cells was below 80-90%, the cells could be resuscitated from the non-plateable state upon temperature upshift and dilution into fresh Brucella-FBP broth. An inhibitory factor was present in the spent medium preventing resuscitation. Further entry into the non-plateable state resulted in loss of cytoplasmic membrane integrity. The maximum 'window of resuscitation' was 3.5 d under a microaerobic atmosphere at 4 °C. Cells incubated microaerobically, or at higher temperatures (20 °C), had a reduced window of resuscitation.

Unlike *Escherichia coli* and *Salmonella typhimurium*, *C. jejuni* does not produce any cold-shock specific proteins as part of an adaptive stress response, at either 32, 25 or 4 °C, as indicated by 2D-PAGE analysis. These results were confirmed by analysing the newly sequenced genome for cold-shock protein homologues. Adaptive stress responses reliant on *de novo* protein synthesis were observed for hydrogen peroxide and trisodium phosphate via analysis of unstressed and stressed 2D-PAGE profiles.

Section 1

Introduction

1.0 Introduction

1.1 General introduction to the genus *Campylobacter*

Campylobacter jejuni belongs to a genus of the Gram-negative, microaerophilic, spiral bacteria which reside in the epsilon subgroup, also containing the genera, *Helicobacter*, *Arcobacter* and *Wolinella* (Table 1.2). *C. jejuni* is termed thermophilic due to its ability to grow at 42°C, a property shared by a number of enteropathogenic *Campylobacter* species (Table 1.1), leading to the general term "thermophilic campylobacters", distinguishing them from the other common species, *C. fetus*, which causes veterinary infections (Tenover & Fennell, 1991).

Campylobacters are highly motile, possessing bipolar flagella, enabling the organism to "cork-screw" through the mucosal surface of the particular epithelial surface the campylobacter frequents (Skirrow, 1990).

The majority of the genus *Campylobacter* are microaerophilic, possessing optimum requirements of 4-5% (v/v) O_2 and 5-10% (v/v) CO_2 (Smibert, 1978). Campylobacters are asaccharolytic, neither fermenting nor oxidising carbohydrates. Energy is obtained via respiration and metabolism of amino acids (Tenover & Fennell, 1991), particularly, serine, proline, aspartate and glutamate (Leach *et al.*, 1997). Campylobacters have small (1.6-1.7 Mbp), AT-rich genomes, with a low GC ratio of 30% (Ketley, 1997). The genome of *C. jejuni* NCTC 11168 has been recently sequenced by the Sanger Centre (http://www.sanger.ac.uk/Projects/C_jejuni/), providing insight into the metabolic and pathogenic capabilities of this organism.

Morphologically, campylobacters are heterogeneous (Section 1.2.6.2), with two states predominating: in younger cultures the rapidly motile, spiral forms predominate, whilst in older/stressed cultures, rapid rounding of the cell occurs

producing predominantly coccoid cultures. This appears to be species dependent (Table 1.1). Coccoid cells are proposed to be dormant, viable forms that may revert to the spiral form (Rollins & Colwell, 1986), leading to the 'viable but-nonculturable' hypothesis being implicated in *C. jejuni* survival and transmission. The hypothesis and relevance to *C. jejuni* will be reviewed later (Section 1.2.8).

Species	Coccoid state	Growth at 42°C	Habitat	Disease †
C. jejuni* subsp *jejuni	+	+	Intestinal tract of man, animals, and poultry	Enteritis, bacteraemia, GBS
C. jejuni* subsp *doylei	+	-	As above	As above, primarily in children
C. coli	+	+	Intestinal tract of pigs and poultry	Enteritis, bacteraemia, GBS
C. lari	+	+	Intestinal tract of seagulls	Enteritis, purulent pleurisy, bacteraemia
C. upsaliensis	+	+	Intestinal tract of cats and dogs	Enteritis, bacteraemia, GBS
C. mucosalis	+	+	Intestinal tract and oral cavity of pigs	Porcine proliferative enteropathy (?), colitis
C. fetus* subsp *fetus	+	-	Ovine and bovine intestinal tracts	Abortion, septicaemia, mainly in sheep and cattle
C. fetus* subsp *venerealis	+	-	Bovine genital tract	Bovine epidemic sterility, abortion
C. hyointestinalis* subsp. *hyointestinalis	-	+	Intestinal tract of cattle and hamsters	Porcine proliferative ileitis, enteritis, proctitis
C. hyointestinalis* subsp. *lawsonii	NK	+	Porcine stomach	NK
C. hyoilei	NK	+	Porcine intestine	Porcine proliferative enteritis
C. sputorum* bvr *sputorum	+	+	Commensal in human gingival crevices	Pulmonary, perianal, groin, and axillary abscesses
C. sputorum* bvr *paraureolyticus	+	+	Commensal on bovine and ovine genitalia	Enteritis
C. sputorum* bvr *faecalis	+	+	Ovine faeces, bovine genitalia	Bovine enteritis?
C. conciscus	NK	+	Human oral cavity and gingiva	Periodontitis, gingivitis, enteritis
C. rectus	NK	-	As above§	As above§
C. curvus	NK	-	As above§	As above§
C. showae	NK	+	As above§	As above§
C. gracilis	NK	NK	As above§	As above§
C. helveticus	-	+	Canine and feline intestine	Canine and feline enteritis
C. hominis	NK	NK	Human intestine	Commensal?

Table 1.1: *Campylobacter* species known to date; GBS = Guillain-Barré Syndrome, NK= not known, † = unless specifically stated the disease is characteristic of humans, § = characteristic as for *C. conciscus*.

Species	Coccoid state	Growth at 42°C	Habitat	Disease †
H. pylori ‡	+	-	Human stomach	Gastric ulcers, gastric cancer
H. cinaedi ‡	+	-	Normal flora of hamsters	Enteritis, proctitis, proctocolitis, in homosexual men
H. fennelliae ‡	+	-	NK	As above
H. westmeadii ‡	NK	-	Blood of AIDS patients	NK
H. heilmanii ‡	+	-	Human, feline and canine stomach	Chronic acute gastritis
H. canis ‡	NK	+	Canine intestine	Canine enteritis, hepatitis
H. salomonis ‡	+	+	Canine stomach	NK
H. bizzozeronii ‡	+	+	Canine stomach	NK
H. felis ‡	+	+	Canine and feline stomach	Canine and feline gastritis
H. colifelis ‡	NK	NK	Feline intestinal tract	Feline enteritis
H. acinonychis ‡	+	-	Cheetah stomach	Cheetah gastritis
H. hepaticus ‡	NK	-	Murine stomach	Murine hepatitis/ liver cancer
H. muridarum ‡	+	-	Rodent stomach	Rodent gastritis
H. rodentium ‡	NK	+	Murine intestine	Commensal?
H. trogontum ‡	+	+	Rodent intestine	Rodent enteritis
H. bilis ‡	+	+	Murine intestine, bile, liver	Murine liver cancer, enteritis
H. cholecystus ‡	+	+	Hamster gall bladder	Hamster pancreatitis, cholangiofibrosis
H. mustelae ‡	+	-	Ferret stomach	Ferret gastritis
H. nemestrinae ‡	+	+	Pig tailed macaque stomach	NK
H. pullorum ‡	+	+	Chickens	Enteritis/ vibrionic hepatitis
H. suncus ‡	+	-	House musk shrew	Shrew gastritis
H. pametensis ‡	+	-	Tern intestine	NK
H. bovis ‡	+	NK	Bovine stomach	Commensal
H. suis ‡	+	NK	Porcine stomach	Porcine gastric ulcer
H. rappini ‡	+	+	Intestine	Bacteraemia, ovine abortion, enteritis
W. succinogenes f	NK	+/-	NK	NK
B. ureolyticus ¶	NK	NK	Genital tract	Non-specific urethritis
An. succiniciproducens #	+	NK	Beagle intestine	Diarrhoea, bacteraemia
A. butzleri §	+ (rare)	+	Porcine intestinal tract	Enteritis
A. cryaerophila §	NK	-	Ovine, porcine and bovine genital tract	Bovine mastitis, abortion in cattle and pigs, bacteraemia
A. skirrowii §	NK	-	As above	Bovine, porcine and ovine abortion
A. nitrofigilis §	+	-	Roots of salt marsh plant	Nitrogen fixation

Table 1.2: Closely related genera to *Campylobacter*. ‡ = *Helicobacter*; § = *Arcobacter*; *f* = *Wolinella*, ¶ = *Bacteroides*, # = *Anaerobiospirillum*; NK = not known; † = unless specifically stated the disease is characteristic of humans

1.1.1 Incidence

Campylobacter jejuni is the most prevalent enteric pathogen in developed countries (Figure 1.1; PHLS web site). The isolation rate in hospitals is 5-6 cases per 10^5 population, however due to the many unreported, mild infections, the true incidence is thought to be 10 cases per 10^3 population (TNACM, 1994). In Britain, the cost per patient is £273, rising to £587 including pain and suffering, producing a yearly figure of £9 million. This figure is proposed to be ten times greater (Skirrow, 1990).

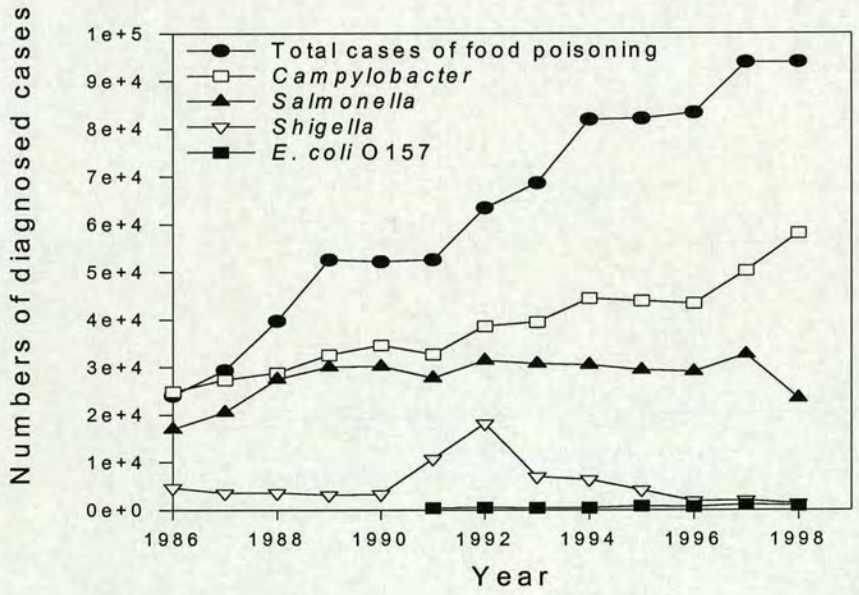

Figure 1.1: Incidences of diagnosed infections of the major enteropathogenic bacteria in England and Wales. Constructed from PHLS data (http://www.phls.co.uk)

1.1.2 Infections

Over 95% of cases of campylobacteriosis are sporadic, the rest attributable to outbreaks, usually due to contaminated private water supplies or unpasteurised milk (Blaser, 1997). The infectious dose ranges from 500 to 10^6 cells, dependent upon strain, and transmission medium (Robinson, 1981, and Saleha *et al.*, 1998).

1.1.2.1 Gastro-enteritis

Campylobacter jejuni primarily causes gastrointestinal infections, clinically presenting as inflammatory enteritis, with an incubation period of 24-72h (Blaser, 1997). Non-specific prodromal symptoms consist of malaise, fever, nausea, myalgia, chills and headache, lasting 24h (Blaser, 1997). Profuse bloody diarrhoea ensues with severe abdominal pain. The illness is usually self-limiting, lasting up to a week (Wallis, 1994, and Allos & Blaser, 1995). The mean excretion time of *C. jejuni* in stools is 16 days (Allos, 1997), extending up to 6 weeks (Nachamkin *et al.*, 1998). Treatment with erythromycin or ciprofloxacin is seldom required (Skirrow, 1990).

Rare abdominal complications may occur, such as massive gastrointestinal haemorrhage, toxic megacolon, pseudomembranous colitis, peritonitis, cholecystitis, proctitis, pancreatitis (Allos & Blaser, 1995), gastritis (Sahay *et al.*, 1995), and appendicitis (Chan *et al.*, 1983). Extraintestinal manifestations occasionally arise as a result of systemic spread via transient bacteraemia due to campylobacter enteritis. Transient bacteraemia may develop into a more fatal bacteraemic manifestation, septic shock, resulting occasionally in acute hepatitis (Korman *et al.*, 1997). Other systemic infections include meningitis, purulent arthritis (Allos & Blaser, 1995), and osteomyelitis (Bracikowshi *et al.*, 1984).

1.1.2.2 Guillain-Barré Syndrome (GBS)

Guillain-Barré syndrome is an acute motor neuropathy of the peripheral nervous system. Demyelination of motor neurones results in paralysis of the regions innervated by these neurones, eventually proving fatal, normally due to respiratory or cardiac failure (Nachamkin *et al.*, 1998). Limb weakness progresses for up to 4 weeks before reaching a plateau (Hughes & Rees, 1997). Substantial recovery from GBS is

usual with administration of human immunoglobulin (Nachamkin *et al.*, 1998), though a mortality rate of 10% exists (Hughes & Rees, 1997). GBS affects 1.3 per 100,000 population world-wide, with males more commonly afflicted than females at a ratio of 3:1. Peaks are observed in young adults and the elderly (Hughes & Rees, 1997 and Nachamkin *et al.*, 1998).

Campylobacter jejuni is the most frequently isolated cause of GBS (41%), particularly from those patients with severe disease and prolonged disability (Hughes & Rees, 1997). One in every 1000 campylobacter infections results in GBS, and one of every 160 *C. jejuni* type O:19 infections (Allos, 1997). The neurologic symptoms occur 1-3 weeks after the onset of diarrhoea (Allos, 1997). Evidence suggests that campylobacter LPS molecules bear a striking resemblance to host neurone (ganglioside) molecules, causing the host immune system to inadvertently attack its own cells (Nachamkin *et al.*, 1998).

1.1.2.3 Reactive arthritis and others

Reactive arthritis is a sterile joint inflammation developed during or after an infection elsewhere in the body, occasionally causing disability (Lichtman, 1996). Other postinfectious complications include haemolytic anaemia, carditis, and encephalopathy (Allos & Blaser, 1995).

1.1.3 Epidemiology of the 'thermophilic' campylobacters

The thermophilic campylobacters are generally transmitted via the faecal-oral route, primarily from the consumption of undercooked poultry or cross-contamination within the kitchen, resulting in sporadic infections (TNACM, 1994). The thermophilic

campylobacters have been isolated from many domestic and wild animals, and therefore the reservoir for infection is large (Skirrow, 1990).

1.1.3.1 Avian reservoir

The most important reservoir of *C. jejuni* are birds. *C. jejuni* typically produces silent infection in avians. The high body temperature (42°C) of birds may explain the high optimal growth temperature of *C. jejuni* (Skirrow, 1990). Thermophilic campylobacters have been isolated from many species of bird, including, gulls, crows, puffins, owls, pigeons (Kapperud & Rosef, 1983), starlings, sparrows, blackbirds (Smibert, 1978), quails (Minakshi & Ayyagari, 1988), turkeys (Wallace *et al.*, 1998), ducks (Saleha *et al.*, 1998), ostriches (Stephens *et al.*, 1998), geese, and cranes (Pacha *et al.*, 1988). In the chicken caecum, densities of 10^{10} cfu/g are not uncommon (Wassenaar *et al.*, 1998), and the entirety of the gut may be colonised (Wallace, *et al.*, 1998). Around 82% of poultry harbour *C. jejuni* in their caeca (Koenraad *et al.*, 1997), and faecal yields can reach 10^7 cfu/g (Wallace *et al.*, 1998). Carcasses are contaminated during slaughter, and remain so when sold at retail (TNACM, 1994).

1.1.3.2 Other livestock as a reservoir of infection

Contaminated milk occurs via faecal contamination at milking (TNACM, 1994), and occasionally through bovine mastitis (Skirrow, 1990). Up to 23% of cattle are infected with *Campylobacter* spp. from drinking contaminated water (Hanninen *et al.*, 1998). Sheep carry *C. jejuni* within the intestine (Stanley *et al.*, 1998) and can survive for up to 4 days in ovine faeces (Jones *et al.*, 1999). Pigs harbour *C. coli* (97% of isolates) more frequently than *C. jejuni* (Munroe *et al.*, 1983), and uncooked

sausage meat is a source of infection (TNACM, 1994). Pig carcass contamination is more frequent than sheep or cattle carcass contamination (Weijtens *et al.*, 1997), although carcass contamination frequently occurs at slaughter in all livestock (TNACM, 1994).

1.1.3.3 Miscellaneous animals

Domestic dogs and cats may harbour *C. jejuni*, with puppies and kittens having higher incidence rates than older animals (TNACM, 1994). Rodents excrete campylobacters in their faeces, and along with birds may represent a means of livestock contamination (TNACM, 1994). Insects, such as, flies (Rosef & Kapperud, 1983), darkling beetles, mealworms (Saleha *et al.*, 1998), and cockroaches (Umunabuicke & Irokanulo, 1986) transiently harbour thermophilic campylobacters, probably due to contact with faecal material.

1.1.3.4 Waterborne and environmental *Campylobacter jejuni*

High carriage rates in domestic and wild animals, ensure large numbers of campylobacters are excreted into the environment, inevitably ending up in waterways due to agricultural run-off or via direct contamination (Arimi *et al.*, 1988 and Koenraad *et al.*, 1997). Treated sewage contains up to 10^3 cfu/L, whilst untreated sewage contains up to 10^7 cfu/L (Arimi *et al.*, 1998 and Höller *et al.*, 1998). Even surface waters frequently (25-95%) harbour campylobacters at a concentration of up to 10^3 cfu/L (Höller *et al.*, 1998). Although large numbers of campylobacters are present in untreated sewage, settlement, filtering and chlorination processes reduce numbers by 99.9% in the final effluent (Arimi *et al.*, 1988). Low temperature and the presence of sediment (nutrients) prolong persistence of *C. jejuni* in the culturable state

in water systems. Light, oxygenation and predation play negative roles in the survival of this organism in aquatic ecosystems (Thomas *et al.*, 1999a,c). Recently the persistent survival of *C. jejuni* in biofilms has been noted (Buswell *et al.*, 1998), with serine addition reducing persistence (Buswell *et al.*, 1999).

Seasonal variation occurs between the numbers of culturable thermophilic campylobacters isolated from sewage effluent. Campylobacter numbers increase during the summer months (May-June), and then decrease during the colder months. The seasonality generally correlated with increased isolation rates from local hospitals, however it was proposed that there was some other reason for the seasonality, perhaps animal faecal contamination (Jones *et al.*, 1990a,c). Conversely, the presence of campylobacters in surface waters showed a negative correlation with community infections. Higher numbers were reported during the winter months, and lower numbers in the summer months, perhaps due to the toxic effect of high sunlight levels (Jones *et al.*, 1990c).

1.1.3.5 Waterborne outbreaks of *Campylobacter* infection

Chickens acquire *C. jejuni* at some stage in their life-cycle, via horizontal rather than vertical transmission (Wallace *et al.*, 1997). The obvious route would be via the faeces of an infected chicken. However, an outbreak of campylobacteriosis in 1986 was traced to a broiler farm, and various mechanisms of flock acquisition were eliminated. Examination of the water supply by fluorescent antibody techniques revealed the presence of campylobacters, however no culturable campylobacters were ever detected (Pearson *et al.*, 1993).

Between 1980 and 1995, 11 waterborne outbreaks of campylobacteriosis were reported in Sweden (Andersson *et al.*, 1997). Waterborne outbreaks have also

occurred in the USA, Finland and the UK, due to contaminated water supplies (Palmer *et al.*, 1983, Aho *et al.*, 1988, and Jones & Roworth, 1996). In many of these outbreaks campylobacters could not be cultured on normal media plates, supporting the hypothesis that *C. jejuni* can enter a dormant, viable but non-culturable 'VBNC' state, and resuscitate under favourable conditions (Rollins & Colwell, 1986).

1.1.4 Campylobacters and the food industry

Campylobacteriosis is a food-borne illness because the bacterium is unable to multiply in food due to its high growth range of 32-47°C (Doyle & Roman, 1981). The vast majority of campylobacter infections are sporadic (>95%) arising via consumption of infected foodstuffs. The following foods have been implicated in the transmission of campylobacters: raw and cooked chicken, game birds, undercooked fish and shellfish (Harris *et al*, 1986).

Campylobacters are sensitive to heating, and are rapidly inactivated upon pasteurisation, and thorough cooking. Inefficient cooking and poor preparatory hygiene result in sporadic cases of campylobacteriosis (TNACM, 1994). Campylobacters survive better under refrigeration than at room temperature (Section 1.2.3.2), remaining culturable on contaminated chickens for up to 17 days at 4°C (TNACM, 1994). A similar time-scale exists for entry into the putative viable but-nonculturable (VBNC) state in *C. jejuni* incubated at 4°C (Jones *et al.*, 1991a). Thus, the existence of a VBNC state has implications for campylobacter transmission on food-stuffs, as well as in aquatic systems.

Campylobacters are sensitive to low water activity and drying; therefore NaCl concentrations above the optimal of 0.5% are inhibitory (TNACM, 1994).

Campylobacters are sensitive to acids, such as lactic acid found in cheese products. (Park *et al.*, 1991). Therefore it is unlikely that salted, pickled, dried, or spiced foods are capable of harbouring infectious campylobacters, at least not in a conventionally detectable state.

1.1.4.1 Poultry products

Poultry are a major source of campylobacters, with up to 48% of all campylobacter enteritis due to the handling and consumption of chickens (Skirrow, 1990). Colonisation of poultry occurs in broiler sheds via horizontal transmission (Wallace *et al.*, 1998). Defeathering, evisceration, and dipping during slaughter lead to contamination of products (Saleha *et al.*, 1998). The processing and packaging of chicken carcasses permits survival of the bacterium on the product. Reduction of thermophilic campylobacter survival on chicken carcasses needs to be undertaken, especially as 28-86% of retail chicken (94% fresh birds; 77% frozen birds) products are contaminated (Park *et al.*, 1991, Moore *et al.*, 1996 and Geilhausen *et al.*, 1996).

1.1.4.2 Milk and other dairy products

C. jejuni can be transmitted in milk, particularly if it is unpasteurised or if birds have pecked at the sealing (Humphrey & Hart, 1988). Milk has a buffering effect on the gastric acid, allowing more organisms to pass through the stomach, and initiate infection (Blaser *et al.*, 1980), indeed an infectious dose of 3 cfu/ml has been reported (Robinson, 1981). Contamination of milk occurs post-pasteurisation because campylobacters are rapidly killed at temperatures above 55°C (TNACM, 1994).

1.1.4.3 Miscellaneous meats

Raw pork, beef, and lamb, occasionally yield campylobacters at a rate of 1.4% (Skirrow, 1990). Cooked meats may also harbour campylobacters, especially if stored at 4°C (Park *et al.*, 1991), however a recent study observed no contamination of pre-cooked foods by thermophilic campylobacters (Moore *et al.*, 1996).

1.2 Survival of bacteria under stress

1.2.1 General introduction

Outside the laboratory environment, bacteria constantly encounter hazards ('stress') which challenge the survival of the organism. To combat these stresses, bacteria have evolved an array of survival mechanisms and stress responses (Table 1.3).

Stress	Stress response	Reference
Starvation/ Stationary phase	RpoS induction: general stress resistance	Kolter *et al.*, 1993
Oxidative shock	Catalase, superoxide dismutase	Demple, 1993
Heat shock	Heat shock proteins (DnaK etc.)	Yura & Nakahigashi, 1999
Cold shock	Cold shock proteins (Csp)	Jones & Inouye, 1994
Osmotic stress (low salt)	Uptake of compatible solutes (betaine etc)	Booth & Louis, 1999
Desiccation (high salt/ drying)	K^+ uptake increases turgor (TrkAEH/ KdpABC)	Booth, 1999
Acid shock	Decarboxylases neutralise acidity	Foster, 1999
Alkaline shock	Na^+/H^+ antiporter and deaminases	Booth, 1999
Antibiotics/ biocides/ detergents	Efflux systems	Paulsen *et al.*, 1996
Heavy metal ions (Cu^{2+}, Hg^{2+})	Specific transporter mechanisms	Silver & Phung, 1996
Organic solvents	Efflux systems	Segura *et al.*, 1999
Radiation	DNA repair enzymes (RecA etc.)	Jeffrey & Mitchell, 1997
Electrophiles (methylglyoxal)	Acidification of cytoplasm via KefB/C	Ferguson, 1999
Organic acids	As acid shock, plus reduction in glutamate pool	Lambert *et al.*, 1997

Table 1.3: Environmental stresses encountered by bacteria and their mechanisms of survival

Some Gram-positive bacteria produce dormant, differentiative forms known as spores (Setlow, 1992 and Gaidenko & Price, 1998), which are resistant to many stresses. Such dormant forms were assumed not to exist in Gram-negative bacteria,

however, the proposed VBNC state has led to great debate on this issue (Section 1.2.8).

1.2.2 Definitions used within the field of microbial stress physiology

The discovery of the 'VBNC' state led to numerous terms being coined to try and bring clarity to the situation of cells which display metabolic activity but are unable to be cultured on normal plating media. This is partly due to the fact that 'VBNC' is a misnomer and an oxymoron (Barer *et al.*, 1993), and also because the standard concept of bacterial viability involves cell replication to an observable level; hence viability of the organism equates with culturability (Kell *et al.*, 1998). This section aims to clarify the terms used within the field and more specifically, those used throughout this text (Table 1.4). Section 1.2.8 discusses the conceptual problems of the VBNC state in more detail.

Term	Definition
Life	In higher organisms, fertility is not the only prerequisite of being alive. In bacteria life is equated with the ability to multiply.
Viable count	Number of bacteria in a sample that can multiply to a **detectable** level. Usually ***plate counts***, but also MPN dilution tubes, or slide culture assays (microcolonies) are used to determine the viable count.
Culturable	Cells may form colonies immediately upon sampling (***immediately culturable***), or require resuscitation (***ultimately culturable***).
Non-plateable	Loss of ability to divide either temporally (injured), prolonged (VBNC/dormant) or permanently (dead) on plates.
Non-culturable	Loss of ability to divide either temporally (injured), prolonged (VBNC/dormant) or permanently (dead) on plates, ***and in broth***.
Viable but non-culturable	Cells which can not be cultured on conventional media but which possess attributes supposedly indicative of life. Implies they can revert to the culturable state.
Active but non-culturable	Demonstration of metabolic processes in non-culturable cells indicative of life.
Dormant	Cells possess little or no activity but are ultimately culturable e.g. spores

Resuscitation	Transition of cells from non-culturable forms to the culturable state. May require specific extracellular signals.
Outgrowth	Growth of a small population of healthy cells present in a population of non-culturable cells, which may give the impression of resuscitation.
Injured	Specific reparative processes are required before the organism can grow on a medium temporally unable to support growth e.g. growth does not occur in the presence of antibiotics, but does in the absence.
Coccoid	Cells less than 2 μm in diameter, occurring in starved/stressed populations, and appear to be the result of rounding of rod-shaped cells (**coccoid transformation**).
Death	Conceptually, cells which were once culturable, and now can not be cultured under any means. Dead cells may be intact and possess residual metabolic activity, therefore being confused with VBNC cells. Only if a cell is lysed can it be truly considered dead in the operational sense.

Table 1.4: Definitions of terms used within the text. Many definitions were adapted from Barer *et al.*, 1993, and Kell *et al.*, 1998

A distinction is required to avoid confusion between viable cells observed by viable counts (plate counts), and cells assumed to be viable due to the retention of metabolic activity or resuscitation from the nonculturable state. In this text, viable cells as determined by plate counts, will be referred to as **plateable cells** and cells that can not form colonies on plates, as **non-plateable cells**, regardless of their physiological status in terms of metabolic activity or the potential to resuscitate. If metabolic activity is observed in non-plateable cells, then this will be stated, and these cells will be equivalent to **'ABNC' cells**. In published studies, the term 'VBNC' will be reserved to describe metabolically active, non-culturable cells which have been shown to resuscitate to the culturable state. Studies on non-culturable cells which show no evidence of resuscitation (yet display activity) will be referred to as 'ABNC' cells, irrespective of the terminology used by the authors.

1.2.3 The cold-shock response in bacteria

Cold-shock causes stabilisation of nucleic acid secondary structure, reducing the efficiency of translation, transcription and DNA replication (Phadtare *et al.*, 1999). Food poisoning organisms are subjected to cold temperatures within refrigerators and freezers at temperatures of 4°C and below. In order to survive, cells have evolved stress mechanisms, including the 'cold-shock' response (Berry & Foegeding, 1998 and Panoff *et al.*, 1998), and entry into the proposed 'VBNC' state (Oliver, 1993; reviewed in section 1.2.8).

1.2.3.1 The cold-shock response in general

When exponentially growing *E. coli* cells are subjected to a temperature downshift from 37 to 10°C, growth ceases for several hours, before a reduced growth rate is established. During the lag phase there is a reduction in saturated fatty acid, causing an increase in membrane fluidity. In conjunction, general DNA, RNA, and protein synthesis is dramatically reduced, and new, 'cold-induced' proteins are synthesised (Jones & Inouye, 1994). One of these comprises up to 13% of the total cellular protein under cold-shock, and was termed, CspA, the major cold-shock protein (Goldstein *et al.*, 1990). CspA is a 7.4 kDa, acidic, cytoplasmic protein, belonging to a family of highly homologous proteins. In *E. coli* there are nine homologues, of which four (CspA, CspB, CspG, and CspI) are cold-shock induced (Etchegaray & Inouye, 1999 and Wang *et al.*, 1999). Homologues have been identified in a broad range of bacteria, including, *Bacillus subtilis* (Graumann & Marahiel, 1999), *Salmonella typhimurium* (Craig *et al.*, 1998), *Pseudomonas fragilis* (Michel *et al.*, 1997) and *Lactobacillus lactis* (Sanders *et al.*, 1999), as well as the eukaryotic organisms, *Dictyostelium discoideum* and *Chlorella vulgaris* (Jones &

Inouye, 1994). It is thought that CspA unwinds RNA molecules folded due to the low temperature. CspA also acts as a cold-shock transcriptional activator of some genes e.g. *hns* and *gyr*A (Jones & Inouye, 1994). CspA expression is regulated transcriptionally, by mRNA instability at higher temperatures (half-lives: 10 sec at 37°C and 30 min at 15°C; Tanabe *et al.*, 1992), and by translational efficiency (Yamanaka *et al.*, 1998). Other cold-shock induced proteins and their functions are shown in Table 1.5.

Protein	Function
Polynucleotide phosphorylase	Degradation of mRNA
NusA	Termination and antitermination of transcription
IF-2α and IF-2β	Initiation of translation
RecA	DNA repair and recombination
Pyruvate dehydrogenase-lipoamide	Decarboxylation of pyruvate
DNA gyrase A subunit	DNA superhelicity
CsdA	mRNA stabilisation
Trigger factor	'Maintenance and repair' function
HscA and HscB	Cold-shock molecular chaperones
LpxP	Lipid A biosynthesis (membrane fluidity)
EF-Tu and EF-G	Translation elongation factors
H-NS	Chromosome condensation
RbfA	Enhances transcriptional capacity of the ribosome

Table 1.5: Some known cold-shock induced proteins of *Escherichia coli*; adapted from Jones & Inouye, 1994, and Jones *et al.*, 1992

Addition of translational inhibitors (chloramphenicol and kanamycin) induces a cold-shock response indicating that the state of the ribosome may be a physiological sensor for induction of the response (Etchegaray & Inouye, 1999). A decrease in translation due to cold-shock triggers a decrease in (p)ppGpp levels, increasing the synthesis of transcriptional, translational and cold-shock proteins (Jones & Inouye, 1994). The function of the cold-shock response is proposed to overcome the translational block resulting from reduced temperature (Jones & Inouye, 1994), as

indicated by the induction of many transcriptional and translational proteins (Table 1.5).

1.2.3.2 The cold-shock response in Campylobacters

Cold-shock in *C. jejuni* can be any temperature below the minimum growth temperature of 31-32°C (Doyle & Roman, 1981 and Hazeleger *et al.*, 1998), the most important of which is refrigeration temperature (4°C). Refrigeration temperature provides protection against the deleterious effects of salt (Doyle & Roman, 1982a and Reezal *et al.*, 1998), atmospheric oxygen levels (Jones *et al.*, 1993), acidic pH (Doyle & Roman, 1981), desiccation (Doyle & Roman, 1982b) and monochloramine (Blaser *et al.*, 1986), experienced at higher temperatures.

C. jejuni survives for extended periods in faeces, bovine milk, water, urine, bile (Blaser *et al.*, 1980), livestock slurry (Easton, 1996), and poultry and other meats (Blankenship & Craven, 1982, and Curtis *et al.*, 1995) when held at refrigeration temperature. In water microcosms, *C. jejuni* (Korhonen & Martikainen, 1991a,b, Terzieva & McFeters, 1991, Fearnley *et al.*, 1996, and Buswell *et al.*, 1998), *C. coli* (Höller *et al.*, 1998), and *C. lari* cells (Thomas *et al.*, 1999a) were observed to survive for longer periods at 4-10°C than at 16-25°C. Overall, low temperatures exert a protective effect to a number of adverse conditions, at least in maintenance of the culturable (presumably infective) state. The investigation of non-culturable states induced under low temperatures is still in its infancy.

Temperature has a profound effect on the induction of the non-culturable state in *C. jejuni* (Boucher *et al.*, 1994) and *C. coli* (Höller *et al.*, 1998). At higher temperatures (20-30°C), entry into the non-culturable state is rapid occurring within days, with conversion to predominantly coccoid (>95%) morphology. Conversely, at

the lower, environmentally important temperatures (4-12°C), entry into the non-culturable state is slow occurring in weeks or months, and the culture remains predominantly spiral (>90%) in morphology (Boucher *et al.*, 1994 and Hazeleger *et al.*, 1995). Investigations into the physiological activity of *C. jejuni* cells revealed that oxygen consumption, catalase activity and ATP generation all occurred at decreased, but detectable rates at low temperature (4°C). Furthermore such cells displayed chemotactic and aerotactic behaviour, whilst no Csp homologues could be detected by PCR (Hazeleger *et al.*, 1998). Therefore, cells have the ability to perform various metabolic processes at temperatures far below those permissive for growth, perhaps influencing environmental transmission (Hazeleger *et al.*, 1998).

1.2.4 Acid and alkali stress in bacteria

1.2.4.1 General acid and alkaline shock in bacteria

Organisms maintain pH homeostasis over a range of external pH conditions by the low proton conductance of the membrane and proton driven transporters (Hall *et al.*, 1995). One of the major obstacles for bacteria to overcome in order to initiate infection is the acidic environment of the stomach. Bacteria such as *S. typhimurium* and *E. coli* can survive pH 2.5 for several hours, if the cells are previously adapted at an intermediate pH. In log phase cells, approximately 70 proteins show altered rates of synthesis when exposed to sublethal acidic pH, in the 'acid tolerance response' (Foster, 1991). Two systems are present in stationary phase cells, one mediated by a RpoS-dependent mechanism (pH-independent), and another which is acid-inducible (Lee *et al.*, 1995), enabling *E. coli* and *Shigella flexneri* to survive for several hours at pH 2.0 (Small *et al.*, 1994). *E. coli* has additional systems operating under fermentative growth requiring low concentrations of arginine or glutamate, inducing

the Adi and GadABC systems respectively (Lin *et al.*, 1995). Amino acid decarboxylases produce alkaline products, neutralising acidic pH, in conjunction with the action of K^+/H^+ antiporters (Hall *et al.*, 1995). A number of virulence factors are pH regulated e.g. ToxR in *V. cholerae*, and PhoP/Q in *S. typhimurium* (Hall *et al.*, 1995). Organic acids e.g. acetate or benzoic acid are used as food preservatives. They acidify the intracellular pH, accumulating as intracellular ions, increasing turgor (Foster, 1999). Approximately 33 proteins are synthesised in response to benzoic acid, including DnaK, GroES and GroEL (Lambert *et al.*, 1997).

Bacteria experience mild alkaline conditions upon passage into the duodenum and jejunum. *E. coli* possesses three Na^+/H^+ antiporters (NhaA, NhaB and ChaA) which accumulate H^+ ions whilst extruding Na^+ ions, decreasing the intracellular pH back towards neutrality. Efflux of sodium ions occurs at neutral pH only for NhaB, whilst NhaA extrudes over the range pH 6.5-8.5, and ChaA over the range pH 7.5-8.5 (Sakuma *et al.*, 1998). NhaA is essential for adaptation to high salinity and alkaline pH in the presence of Na^+ ions, and transcription is induced by Na^+ ions, and regulated by NhaR (Dover *et al.*, 1996). NhaA activity increases by 1000-fold with an upshift from pH 7.0 to 8.0 (Hall *et al.*, 1995). The action of deaminases also decreases the intracellular pH due to the production of acidic products (Hall *et al.*, 1995). Alkaline shock in *Enterococcus faecalis* resulted in the synthesis of 37 stress proteins, including DnaK and GroEL (Flahaut *et al.*, 1997).

A number of pH-inducible genes have been identified (Table 1.6).

Protein	Function	Induction conditions
CadAB	Lysine decarboxylase	Acid
GadABC	Glutamate decarboxylase	Acid/ Alkaline with anaerobiosis
AceA	Isocitrate lyase	Acid
OmpA, OmpC, OmpF	Porins	Acid
MalE	Maltose binding protein	Alkaline
SurA	Periplasmic foldase	Alkaline
TnaA	Tryptophan deaminase	Alkaline
NhaA	Na^+/H^+ antiporter	Alkaline
AhpC	Alkylhydrogenperoxide reductase	Acid
ManX/PtsH	Phosphotransferase system	Acid
Adi	Arginine decarboxylase	Acid
GatY	Galactitol fermentation enzyme	Acid
PhoP/Q	Aids survival in macrophages	Acid
YfiD	Pyruvate formate lyase homologue	Org
MarA, Ina	NK	Org
GroEL, DnaK, GrpE, HtpG	Molecular chaperones	Org, Acid, Alkaline
CydAB	Cytochrome *d*-type oxidase	Acid
ProU	Glycine betaine transport	Acid

Table 1.6: Identified proteins induced by pH upshift or downshift. Adapted from Hall *et al.*, 1995, Foster, 1991, Lambert *et al.*, 1997, Blankenhorn *et al.*, 1999 and Jordan *et al.*, 1999. Org = organic acid, NK = not known.

1.2.4.2 Acid and alkaline shock in *Campylobacter*

The pH range for growth is 5.5 to 8.0. The death rate at pH 3.0 to 4.5 was temperature dependent, with survival longest at the lower temperature (4°C) (Doyle & Roman, 1981). At very low pH values (3.0-3.5), *C. jejuni* was readily inactivated at all temperatures (Doyle & Roman, 1981). At even lower pH values there was a 6 log decrease in cells within 30 mins at pH 2.3-2.5 (Blaser *et al.*, 1980). In comparison to other organisms, *C. jejuni* is 1000-fold more sensitive to acidic conditions than *S. typhimurium*, *E. coli* and *Shigella flexneri*, but is 100-fold more resistant than *V. cholerae* (Waterman & Small, 1998).

Acid and oxidative shock would be experienced by *C. jejuni* when taken up by macrophages within the phagocytic vesicle. Upon internalisation by INT407 cells, 14

proteins were synthesised (Konkel & Cieplak, Jr, 1992), perhaps aiding survival in the acidic environment of the phagolysosome and the stomach. Internalisation of *C. jejuni* into phagocytic vesicles by Hep-2 cells results in a lysosomal response, causing morphological conversion from the spiral to the coccoid form, correlating with a decrease in viability (de Melo *et al.*, 1989).

The protein response to pH shock was characterised in *C. jejuni*. Two proteins of 45 and 64 kDa, corresponding to *E. coli* GroEL and GroES homologues were expressed in heat (60°C) and alkaline (pH 8.6) shocked cells. Additionally, four proteins of 22, 38, 53, and 66 kDa were induced by pH 8.6. Acid-shock did not induce any proteins (Wu *et al.*, 1994).

1.2.5 Oxidative shock in bacteria

1.2.5.1 General oxidative shock in bacteria

Oxygen exerts its toxic effects indirectly, via the formation of reactive oxygen intermediates (ROIs), such as hydrogen peroxide, hydroxyl radicals, superoxide anions, and singlet oxygen (Farr & Kogoma, 1991). Hypochlorous acid, used in chlorination and produced as part of the oxidative burst in macrophages, can generate ROIs (Dukan & Touati, 1996). ROIs are formed by photo-oxidation of media components, such as, amino acids, within phagolysosomes and by the action of the cellular electron transport chain, peroxidising lipids and damaging nucleic acids and many proteins including, cytochromes, flavoproteins, Fe/S proteins and TCA enzymes (Farr & Kogoma, 1991, Iuchi & Weiner, 1996, and Miller & Britigan, 1997).

In *E. coli*, superoxide anions are scavenged by three different superoxide dismutases (SodA, SodB and SodC), whilst hydrogen peroxide is scavenged by two

catalases (KatE and KatG) and alkylhydroperoxide reductase (AhpCF) reduces organic hydroperoxides. The regulation of these protective enzymes and other proteins involved in the protective response are under the control of at least four interacting regulatory systems: OxyR, SoxRS, ArcAB, and Fnr (Iuchi & Weiner, 1996).

Approximately 30 proteins are induced by sublethal concentrations of hydrogen peroxide and hypochlorous acid, of which nine are induced by the regulatory protein, OxyR, including, KatG, AhpCF, DnaK, RecA, GroEL and glutathione reductase (*gor*) (Morgan, *et al.*, 1986, Dukan & Touati, 1996, and Farr & Kogoma, 1991). A separate regulon of at least 10 proteins is induced in response to superoxide anions by the SoxRS two-component regulatory system. SoxRS induces SodA, endonuclease IV, glucose-6-phosphate dehydrogenase, NADH dehydrogenase, GroEL, GroES, fumarase C, acinotase A operon, and NADH:ferredoxin oxidoreductase. At least a further 20 proteins are induced independently of SoxRS by superoxide anions (Walkup & Kogoma, 1989 and Farr & Kogoma, 1991). The ArcAB system operates under anaerobic conditions, activating 9 operons, and repressing a further 17 operons (Iuchi & Weiner, 1996). Cells also possess non-enzymatic antioxidants such as glutathione and thioredoxin, with glucose-6-phosphate dehydrogenase generating NADPH as an electron source for thioredoxin reductase and glutathione reductase (Farr & Kogoma, 1991). Oxidative DNA damage is prevented by the action of RecA, RecBC nuclease, exonuclease III, exinuclease ABC (UvrABC), DNA glycosylase and DNA polymerase I and III (Farr & Kogoma, 1991).

1.2.5.2 Oxygen: a deadly toxin to *Campylobacter jejuni*

Campylobacters are microaerophilic organisms requiring oxygen (3-10% v/v) as the terminal electron acceptor, but displaying sensitivity to atmospheric oxygen levels (Hoffman *et al.*, 1979a). Addition of various supplements that quench the formation of ROIs offer protection (Hoffman *et al.*, 1979a,b, Moran & Upton, 1986, Moran & Upton, 1987a,b, and Boucher *et al.*, 1994). Such supplements, including FBP (Hoffman *et al.*, 1979a,b, and Chou *et al.*, 1983), and charcoal (Bolton *et al.*, 1984), prolong maintenance of the culturable, spiral state (Chou *et al.*, 1983). Aerated, stressed *C. jejuni* cells can be protected from loss of plating ability by at least two days, via incubation in the presence of a block of beechwood, dependent on block thickness (Boucher *et al.*, 1998), leading the author to question the use of wooden cutting boards for preparing poultry meat.

Aeration rapidly promotes transformation to the coccoid, 'VBNC' state (Moran & Upton, 1986, Boucher *et al.*, 1994 and Harvey & Leach, 1998). Cultures aerated in the presence of light transform to the coccoid state more rapidly than those aerated in the dark, whereas cultures incubated microaerobically in the light and dark turned coccoid at the same rate, but at a slower rate than in the corresponding aerated cultures (Moran & Upton, 1987b).

C. jejuni can adapt to growth in aerobic atmosphere (Jones *et al.*, 1993, Harvey & Leach, 1998 and Chynoweth *et al.*, 1998) with retention of pathogenicity (Jones *et al.*, 1993) and spiral morphology (Harvey & Leach, 1998), but survival was not enhanced over unadapted cells incubated in stream water or chicken products (Chynoweth *et al.*, 1998). Furthermore, oxidative stress increases invasiveness and intracellular survival (Harvey *et al.*, 1996). Protection against both loss of viability

and coccoid formation are elicited by addition of catalase and superoxide dismutase (SOD) to the media (Moran & Upton, 1987a, and Boucher *et al*, 1994). It is known that *C. jejuni* possesses catalase (Grant & Park, 1995), Fe-SOD (Purdy & Park, 1994), GroEL (Takata *et al.*, 1992) and alkyl hydrogenperoxide reductase (AhpC; Baillon *et al.*, 1999), all involved in protection against oxidative damage. These proteins are known to protect against the effects of oxidative stress in *Escherichia coli* (Demple, 1993), *Salmonella typhimurium* (Morgan *et al.*, 1986) and *Bacteroides fragilis* (Rocha *et al.*, 1996). Recently PerR has been shown to regulate expression of AhpC and KatA in *C. jejuni*, supposedly compensating for the absence of OxyR homologues (van Vliet *et al*, 1999). Interestingly, the specific activity of SOD in coccoid cells, was 44% less than in spirals, hence it is predicted that coccoid forms are more susceptible to superoxide anions (Moran & Upton, 1987a).

1.2.6 Starvation and stationary phase in bacteria

1.2.6.1 General stationary phase in Gram-negative organisms

Stationary phase is that stage in a batch culture where nutrient depletion occurs, causing a decrease in growth rate to zero, resulting in no net gain or decrease in cell numbers. However, far from being a static phase, many dynamic processes are occurring, and the physiology of stationary phase cells depends on the conditions that resulted in growth cessation (Huisman *et al.*, 1996).

Upon entry into stationary phase there are often accompanying changes in cell morphology. Cells reduce in size from the usual rod-shape to smaller, more spherical cells (coccoid; ultramicrocells), increasing the surface to volume ratio (S/V) and contributing to sequestration of low concentrations of nutrients (Siegele & Kolter, 1992 and Kjelleberg *et al.*, 1993). The peptidoglycan is thicker and more cross-linked,

increasing protection against penicillins (Siegele & Kolter, 1992 and Huisman *et al.*, 1996). Starved cells obtain resistance to lethal doses of heat, hydrogen peroxide, hyperosmotic stress, acid and disinfectant agents, due to the induction of general stress proteins (Tables 1.7 and 1.8; Huisman *et al.*, 1996). The metabolic rate decreases, but some level of endogenous metabolism remains, allowing maintenance of ATP at a reduced level, and the protonmotive force (pmf) across the membrane (Siegele & Kolter, 1992). Starved cells become hydrophobic and more adhesive, due to the production of surface structures. Aggregates form due to secreted attractants sensed by chemotactic machinery (Siegele & Kolter, 1992), perhaps aiding survival.

Entry into the stationary phase induces changes in the fatty acid composition of the cytoplasmic membrane. There is a reduction in membrane fluidity as monounsaturated fatty acids are converted to their cyclopropyl derivatives via cyclopropyl fatty acid synthase (Siegele & Kolter, 1992). Energy reserves of glycogen, poly-β-hydroxybutyrate, and polyphosphate are gradually utilised upon starvation (Matin, 1992 and Huisman *et al.*, 1996). A number of metabolites are excreted into the medium, e.g. acetate, and reduced glutathione. *E. coli* cultures also release homoserine lactone which can induce RpoS, and may be a general starvation signal for entry or perhaps exit from starvation-induced survival (Huisman *et al.*, 1996).

DNA remains stable in most bacteria even during prolonged starvation. The chromosome undergoes topological changes consistent with reduced gene expression (Kolter *et al.*, 1993). The nucleoid condenses resulting from increased amounts of the histone-like proteins, H-NS, IHF and Dps, furthermore cells possess one chromosome, compared to four in exponential cultures (Kolter *et al.*, 1993). RNA is

rapidly degraded during starvation in *E. coli*. Ribosomal RNA is preferentially degraded, resulting in a reduction in the ribosomal machinery, and therefore protein synthesis (Matin, 1992), unlike the situation in *Vibrio* spp where degradation of ribosomes is very slow (Kjelleberg *et al.*, 1993).

At the onset of stationary phase the rate of protein synthesis is reduced, with the rate dropping to 0.05% after 11d (Kolter *et al.*, 1993 and Kjelleberg *et al.*, 1993). Concomitantly, protein degradation increases five-fold upon entry into stationary phase. Protein degradation provides amino acids for the synthesis of proteins conducive to starvation and stress survival (Siegele & Kolter, 1992).

In *E. coli* a core set of 15-30 proteins are induced, and these are temporally expressed in *E. coli*, *S. typhimurium* and *Vibrio* spp (Kolter *et al.*, 1993). Of the starvation-induced genes, two-thirds require cAMP for induction. Such proteins are known as Cst (carbon starvation) proteins, whilst those expressed in a cAMP-independent manner are termed Pex (post-exponential) proteins (Matin, 1992). Many starvation-induced proteins are transcriptionally regulated by a starvation-induced sigma factor (RpoS). At least 30 proteins require RpoS for expression during starvation. Table 1.7 contains a selected list of the *E. coli* RpoS-dependent genes and their respective functions. RpoS itself is subject to extensive regulation at the transcriptional, translational and post-translational level by a number of proteins, including HN-S (Huisman *et al.*, 1996).

Gene	Function
*app*Y, *cyx*AB	Anaerobic respiration
*bol*A, *fic*A	Cell-shape determination
*csg*A	Curli fibronectin binding fibres; starvation aggregation factor
cfa	Maintenance of membrane function
*kat*E, *gor*, *dps*, *xth*A	Resistance to oxidative stress
mcc	Microcin C7; antibiotic
*ots*AB, *osm*B, *pro*P, *pro*U, *tre*A	Resistance to osmotic stress

Table 1.7: Selected RpoS-dependent genes, and their functions (Huisman *et al.*, 1996)

Induction of approximately 20 proteins induced by carbon starvation is rpoS-independent (Table 1.8; Kolter *et al.*, 1993). Starvation results in a *rel*A-dependent and *rel*A-independent (via SpoT) increase in ppGpp, inducing the stringent response. Regulation of many genes, including increasing RpoS levels, is mediated by ppGpp (Huisman *et al.*, 1996). Other regulatory genes that respond to starvation or regulate starvation-inducible genes include *usp*A, *mpr*A, and *csr*A, the carbon storage regulator (Huisman *et al.*, 1996).

Gene/protein	Function	Regulators
*acr*AB	Multidrug efflux pump: hydrophobic inhibtor resistance	
*cst*A	Peptide transport system in glucose starvation	cAMP-CRP
*cyd*AB	Cytochrome *b* oxidase	*arc*AB
DnaK, GroEL, HtpG	Heat-shock proteins: prevent premature folding of nascent polypeptides	
*glg*CAB	Glycogen biosynthesis	cAMP-CRP, ppGpp
Lrp	Leucine responsive regulatory protein	ppGpp
*osm*C	Protection against osmotic stress	
psp	Prevents dissipation of proton-motive force	σ^{54}
rmf	Ribosome dimerisation	

Table 1.8: Selected RpoS-independent genes, their functions and regulatory mechanisms (Huisman *et al.*, 1996)

Stationary phase cells respond rapidly to the addition of fresh nutrients, unlike Gram-positive spores that require activating agents (Kolter *et al.*, 1993 and Kjelleberg *et al.*, 1993). Macromolecular synthesis occurs in differing temporal patterns. RNA synthesis starts almost immediately whilst protein synthesis lags for a short time

(Kolter *et al.*, 1993). Cells then increase in mass, DNA synthesis starts, and finally the cells multiply. Cells lose their enhanced resistance to various environmental challenges (Kolter *et al.*, 1993). Approximately 20 starvation induced proteins are repressed upon nutrient addition, and a number of new proteins are temporally synthesised. In *E. coli*, Fis (non-specific DNA binding protein), ClpP, CydAB, RelA, RelB, H-NS, RpoS and SurB (cytochrome oxidase biosynthesis), are presently the only proteins known to be induced (Kolter *et al.*, 1993 and Östling *et al.*, 1997).

1.2.6.2 Stationary phase in Campylobacters

When *C. jejuni* is grown in complex media under optimal conditions, various morphological changes are observed. After 12h incubation (mid-log phase) all the cells were short spirals (Griffiths, 1993). After 24h (early stationary phase; slow growth) incubation all cells were 100% plateable spirals (Ng *et al.*, 1985), twice the length of log phase spirals (Griffiths, 1993, Leach *et al.*, 1997 and Thomas *et al.*, 1999b). By 48h (late stationary phase) the culture was a heterogeneous population of coccoid cells and spirals 3-4 times the length of log phase cells (Griffiths, 1993 and Thomas *et al.*, 1999b). Continued incubation resulted in a largely coccoid population that showed extensive clumping (Ng *et al.*, 1985, Griffiths, 1993 and Thomas *et al.*, 1999b). After 5-12 days incubation spirals (10^5-10^6 cells ml^{-1}) were still present in predominantly coccoid (10^9 cells ml^{-1}) cultures (Ng *et al.*, 1985, and Bovill & Mackey, 1997). No difference in the protein profiles of any morphological form was observed (Thomas *et al.*, 1999).

Entry into stationary phase involves changes at the morphological and molecular level in *E. coli*, and *Vibrio spp* (Section 1.2.6.1). Some of these changes have been observed in campylobacters entering the stationary phase. *E. coli* and

Vibrio spp cells reduce in size upon entering stationary phase (Matin, 1993, and Kjelleberg *et al.*, 1993); it is well documented that campylobacters form coccoid cells under adverse conditions, and indeed during stationary phase (Griffiths, 1993). Stationary phase cells adhere due to the production of fimbriae and special adhesins (Matin, 1992); it has been observed that campylobacters clump extensively when in the coccoid state (Boucher *et al.*, 1994), furthermore, campylobacters have recently been shown to produce fimbriae (Doig *et al.*, 1996). Low growth rates in continuous culture promoted conversion of membrane fatty acids to their cyclopropane derivatives, indicative of maintenance of membrane fluidity observed in stationary phase cells (Leach *et al*, 1997). Condensation of the chromosome occurs in *E. coli* (Matin, 1992) and *C. jejuni* has been shown to possess a histone-like protein (Hup) that is proposed to aid condensation of the chromosome (Konkel *et al.*, 1994). Presently no evidence of stationary phase regulated genes or specific sigma factors have been published.

1.2.7 Miscellaneous stresses and their effect on Campylobacters

The heat-shock response of *C. jejuni* has been recently characterised by 2D-PAGE, with 24 proteins upshifted in response to a temperature increase from 37 to 46°C (Konkel *et al.*, 1998). Some of these have been identified, including GroES, GroEL, ClpB, DnaK (Theis *et al.*, 1999a,b,c), and DnaJ (Konkel *et al.*, 1998). Cell injury occurs upon heating at 46°C for 45 min, evidenced by leakage of nucleic acids. Addition to the medium of NH_4Cl, KCl or $LiCl_2$ prevented injury. Heat damage could be repaired within 4h in fresh broth at 42, but not 5°C (Palumbo, 1984).

C. jejuni is sensitive to sodium chloride concentrations above 1.5% at 25-42°C, and 1% at 4°C, however, survival was prolonged at the lower temperature

(Doyle & Roman, 1982a). *C. jejuni* shows marked sensitivity to changes in osmolality of the medium (Lowrie *et al.*, 1974 and Reezal *et al.*, 1998). *C. jejuni*, *C. coli* or *C. lari* populations declined and turned coccoid in low osmolarity media (below 130 mOsm) independent of temperature. Coccoid formation occurred regardless of the osmolyte used. However, at high osmolarities at which growth was not observed (above 175 mOsm), the decline was less pronounced at lower temperatures and the cells were rod-shaped (Reezal *et al.*, 1998). Dried *C. jejuni* cells can survive in the culturable state on surfaces for weeks, especially at low temperature (4°C). Survival is increased if drying occurs in the presence of broth rather than skimmed milk. This has implications for the poultry industry as campylobacters are often spread onto surfaces during the evisceration process (Doyle & Roman, 1982b).

C. jejuni has limited survival at 0.1 mg/L free chlorine, with a 99% reduction within 15 min, hence standard disinfection procedures are deemed adequate for the elimination of this pathogen from water supplies (Wang *et al.*, 1983 and Blaser *et al.*, 1986). Exposure (5 min) of culturable and non-culturable (coccoid) cells to industrial concentrations of disinfectants (chlorine, quaternary ammonium compound and amphoteric based) resulted in large reductions in viability (based on detection of esterase activity by flow cytometry). Generally culturable cells were more resistant than non-culturable cells, except in the case of chlorine, raising the possibility of these forms being present after disinfection processes used within the food industry (Rowe *et al.*, 1998).

Trisodium phosphate (TSP) is being introduced as an alternative to chlorine treatment for washing poultry carcasses. TSP at a 10% (w/v) working solution reduces *C. jejuni* levels on chicken carcasses by up to 1.5 log units (Slavik *et al.*, 1994).

Application of an electrical current destroyed *C. jejuni* in poultry chiller water, and the rate was increased with salt concentration (TSP or NaCl) and pH increments (Li *et al.*, 1995). TSP also reduces the loads of *Salmonella* spp, *L. monocytogenes*, and *S. aureus* (Hwang & Beuchat, 1995). Irradiation is widely used in the food and water industries (Farkas, 1998). *C. jejuni* is more susceptible to UV (Butler *et al.*, 1987) and γ-irradiation (Clavero *et al.*, 1994), than *E. coli* and *Salmonella*, and the decline rate is independent of temperature (Farkas, 1998).

1.2.8 The viable but-nonculturable (VBNC) hypothesis

1.2.8.1 General introduction to the VBNC state

Large numbers of bacteria enter marine and aquatic environments by surface drainage and sewage outfall, but a rapid decrease in conventionally culturable bacteria is observed. Suggested factors for this phenomenon include dilution, sedimentation, predation, toxic compounds, hydrostatic pressure, solar radiation, temperature, salinity, oxidation-reduction potential, nutrient availability and entry into a putative 'VBNC' state (McDougald *et al.*, 1997, Barcina *et al.*, 1997 and Troussellier *et al.*, 1998). In the early 1980's, marine bacteriologists noted that certain bacteria, particularly *Vibrio* species (Xu *et al.*, 1982), lost culturability when stressed, yet the total count remained constant (Figure 1.2). Assays were developed enabling the researcher to determine whether a cell was metabolically active. It was observed that the 'ABNC' population closely followed the total count, demonstrating that cells remain metabolically active, and potentially viable, despite being nonculturable. The existence of such a state in pathogenic bacteria questions the value of culture based methods for public safety (Roszak & Colwell, 1987a).

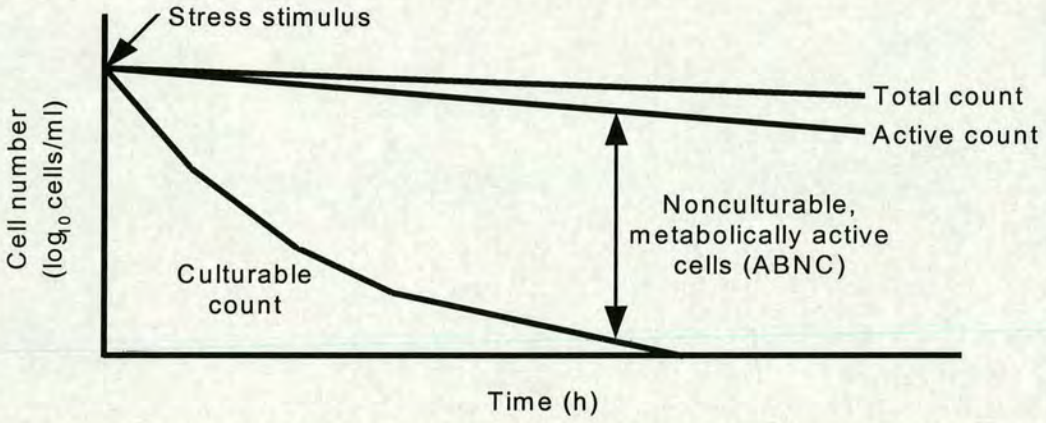

Figure 1.2: General characteristics of a population of stressed cells as a function of time

A variety of conditions have been implicated in the induction of the 'ABNC' state in various bacterial species, such as cold-shock (Smith *et al.*, 1994), starvation (Bovill & Mackey, 1997), oxidative shock (Arana *et al.*, 1992), osmotic stress (Roth *et al.*, 1988), alkaline shock (Bode *et al.*, 1993), aerosolisation (Heidelberg *et al.*, 1997), antibiotics (Nilius *et al.*, 1993), and chemical stress by Cu^{2+} ions (Alexander *et al.*, 1999), hypochlorous acid (Dukan *et al.*, 1997), or hypochlorite ions (Bej *et al.*, 1991). However, researchers often use systems referred to as 'microcosms' which refer to *in vitro* systems mimicking aquatic or marine environments. It should be noted that these systems subject the cells to multiple stresses, such as starvation, cold-shock, oxidative shock, and osmotic shock, as well as physicochemical factors specific to the water source. Hence, it is difficult to state that one stress resulted in the formation of the 'ABNC' state in such a study. Nevertheless, a number of bacteria have been shown to enter such a state (Table 1.9) as a result of numerous stresses.

Species	Gram stain	Size reduction	Resuscitate from VBNC state	ABNC	Research group
Escherichia coli	-	+	+	+	Rigsbee *et al.*, 1997
Salmonella enteritidis	-	+	+	+	Roszak *et al.*, 1984
Salmonella typhimurium	-	+	ND	+	Baleux *et al.*, 1998
Salmonella typhi	-	ND	ND	+	Cho & Kim, 1999
Salmonella salamae	-	+	ND	+	Monfort & Baleux, 1994
Shigella sonnei	-	ND	-	+	Colwell *et al.*, 1985
Shigella flexneri	-	ND	-	+	Colwell *et al.*, 1985
Shigella dysenteriae	-	+	-	+	Rahman *et al.*, 1994
Yersinia ruckeri	-	+	+	+	Romalde *et al.*, 1994
Yersinia enterocolitica	-	ND	ND	+	Smith *et al.*, 1994
Yersinia pseudotuberculosis	-	ND	ND	+	Buzoleva *et al.*, 2000
Klebsiella pneumoniae	-	+	ND	+	Diaper & Edwards, 1994
Klebsiella planticola	-	ND	ND	+	Heidelberg *et al.*, 1997
Enterobacter aerogenes	-	ND	ND	+	Byrd *et al.*, 1991
Serratia marcescens	-	ND	ND	+	Heidelberg *et al.*, 1997
Vibrio cholerae	-	+	+	+	Wai *et al.*, 1996
Vibrio vulnificus	-	+	+	+	Whitesides & Oliver, 1997
Vibrio fischeri	-	ND	ND	+	Lee & Ruby, 1995
Vibrio harveyi	-	ND	ND	+	Duncan *et al.*, 1994
Vibrio anguillarium	-	+	ND	+	Nelson *et al.*, 1997
Vibrio parahaemolyticus	-	+	ND	+	Pace *et al.*, 1997
Aeromonas salmonicida	-	+	+	+	Effendi & Austin, 1995
Aeromonas hydrophila	-	+	ND	+	Morgan, *et al.*, 1991
Campylobacter jejuni	-	+/-	+?	+	Bovill & Mackey, 1997
Campylobacter coli	-	+	ND	+	Höller *et al.*, 1998
Helicobacter pylori	-	+	+	+	Wang *et al.*, 1997
Prolinoborus fasciculus	-	+	ND	+	Koechlein & Kreig, 1998
Pseudomonas aeruginosa	-	ND	+	ND	Lueng *et al.*, 1995
Pseudomonas syringae	-	+	ND	+	Wilson & Lindow ,1992
Pseudomonas putida	-	+	+	+	Totemeyer *et al.*, 1996

Pseudomonas fluorescens	-	+	ND	ND	van Overbeek *et al.*, 1995
Xanthomonas campestris	-	ND	-	+	Ghezzi & Steck, 1999
Pasteurella piscicida	-	ND	+	+	Margarinos *et al.*, 1997
Legionella pneumophila	-	+	+	+	Steinert *et al.*, 1997
Agrobacterium tumefaciens	-	-	ND	+	Manahan & Steck, 1997
Rhizobium meliloti	-	-	ND	+	Manahan & Steck, 1997
Alcaligenes eutrophus	-	ND	ND	+	Byrd *et al.*, 1991
Cytophaga allerginae	-	ND	ND	+	Heidelberg *et al.*, 1997
Xenorhabdus nematophilus	-	ND	ND	+	Morgan, *et al.*, 1997
Photorhabdus luminescens	-	ND	ND	+	Morgan, *et al.*, 1997
Photobacterium damselae	-	+	+	+	Fouz *et al.*, 1998
Francisella tularensis	-	ND	ND	+	Henningson *et al.*, 1998
Listeria monocytogenes	+	ND	ND	+	Bremer *et al.*, 1998
Lactobacillus plantarum	+	ND	+	+	Muller & Seyfarth, 1997
Micrococcus flavus	+	ND	ND	+	Byrd *et al.*, 1991
Lactococcus lactis	+	ND	+	+	Muller & Seyfarth, 1997
Enterococcus faecalis	+	ND	ND	+	Byrd *et al.*, 1991

Table 1.9: Bacterial species that enter the putative viable but non-culturable state; ND = not determined; ABNC = active but non-culturable, the cells have been shown to retain certain metabolic activities; ? = resuscitation in *Campylobacter jejuni* is subject to much debate (Section 1.2.8.7)

In most genera studied, VBNC cells show a dramatic size reduction, often being smaller than starved cells. The ribosomal machinery and nucleic acids are present in reduced amounts, concomitant with a reduction in macromolecular metabolism (Oliver, 1993). Generally, increased amounts of DNA are required for PCR detection of VBNC cells, as the majority have reduced nucleic acid contents (Weichart *et al.*, 1997). Starved cells respond rapidly to reversal of the inducing factor (i.e. carbon limitation), whilst it usually takes hours or days in the case of VBNC cells. VBNC cells differ from classically injured cells in their inability to grow on either selective or non-selective media (Oliver, 1993). The incubation temperature and physiological age of the cells influence the non-culturable response of many organisms. RpoS induction prolongs survival in the culturable state (Munro *et al.*, 1994 and Gourmelon *et al.*, 1997), explaining observations that stationary phase cells survive longer than exponential phase cells in microcosms and inferring that starvation represses the VBNC response (Oliver, 1993).

The existence of a 'VBNC' state has implications for the food, medical and water industries, because present culture-based methods are inadequate for the detection of this putative physiological state (McKay, 1992). It has been suggested that non-culturable cells express virulence factors (Pommepuy *et al.*, 1996 and Rahman *et al.*, 1996), and can revert to a culturable state *in vivo* (Oliver & Bockian, 1995 and Colwell *et al.*, 1996; Section 1.2.8.4). Indeed, plasmids have been shown to be maintained in non-culturable cells for prolonged periods (Byrd & Colwell, 1993 and Arana *et al.*, 1997), raising the possibility of genetic exchange between such cells and their culturable counterparts.

1.2.8.2 Conceptual problems with the 'viable but non-culturable' state

The classical definition of bacterial viability equates with culturability (replicative ability). In higher organisms, although such terminology exists it is incorrect. The ability to multiply (fertility) is not synonymous with being alive. Yet this assumption provides the definition for viability in microbiology (Kell *et al.*, 1998). This is erroneous as bacteria enter states from which they exhibit no signs of life yet they can return to a physiologically active state, e.g. spores (Kell *et al.*, 1998).

It has been experimentally determined that populations of metabolically active cells exist that do not form colonies on agar plates (Figure 1.2). As they initially exhibit no 'viable count' they were termed 'viable but non-culturable' (Roszak & Colwell, 1987a). The definition of the VBNC state has been questioned as an oxymoron and a misnomer because by definition their viability can not be directly demonstrated (Barer *et al.*, 1993 and Kell *et al.*, 1998). This, in conjunction with a failure to clearly differentiate between 'viability' and 'culturability', has exacerbated the problems associated with the VBNC concept (Barer, 1997 and Kell *et al.*, 1998).

It has been proposed that the VBNC state represents a form of dormancy induced by environmental stimuli and/or nutrient deprivation (Roszak & Colwell, 1987a); however, dormancy refers to cells with negligible activity but which are ultimately culturable. VBNC cells are the opposite, being metabolically active but 'non-culturable', hence the term **active but non-culturable (ABNC)** has been proposed (Kell *et al.*, 1998). Non-culturable bacteria that have not been resuscitated should not be considered to be viable, as their only feature of life is the retention of metabolic activity, which may or may not indicate viability (Barer *et al.*, 1993 and

Kell *et al.*, 1998). The performance and interpretation of assay techniques (Section 1.2.8.3) and methods of recovering VBNC cells (Section 1.2.8.4) remains a central issue (Barer *et al.*, 1993). Therefore, assignment to a VBNC state for a particular species is only as good as the activity and recovery assays used and their interpretations (Kell *et al.*, 1998), for example, early studies into this state used acridine orange staining to distinguish between different physiological states. It is now known that many different physical parameters other than cellular status affect the colour stain, perhaps producing misleading results (Section 1.2.8.3.3).

The purpose of direct assays is to detect physiological activity and integrity of non-culturable cells (Barer *et al.*, 1993), however, all approaches for assaying viability are undermined by an uncertainty of the minimum requirement for defining cell viability (Barer *et al.*, 1993 and Kell *et al.*, 1998). In microbial physiology the situation is not clear-cut. For example, spores fail most activity assays (yet are alive), however, anucleate minicells (incapable of replication) retain metabolic activity giving positive results in most activity assays (Barer *et al.*, 1993). Furthermore, viable cells may only divide a few times, not enough to form a colony (microcolony), but can be visualised microscopically on slide culture (Torrella & Morita, 1981). Support from convincing resuscitation data is required to demonstrate the validity of the activity assay in designating cells as VBNC (Kell *et al.*, 1998). Most studies fail to discriminate between recovery and the regrowth of residual culturable cells, and in most cases where dilution of non-culturable cells was sufficient to statistically remove any viable cells, recovery was unsuccessful (Kell *et al.*, 1998). A single protocol for resuscitation of all non-culturable cells is unlikely, rather species or strain-specific protocols will need to be devised (Kell *et al.*, 1998).

It has been proposed that there may be two fates for ABNC cells. One is the transition to a VBNC state, with loss of culturability and maintenance of cell integrity and intact nucleic acids, whilst the second involves progressive loss of cellular integrity and degradation of nucleic acids leading to loss of viability (McDougald *et al.*, 1998). The latter transition infers that injured, transiently non-culturable cells would develop (Barer *et al.*, 1993), raising the possibility that resuscitation is recovery from injury (Dukan *et al.*, 1997 and Kell *et al.*, 1998). VBNC cells should be recoverable after prolonged periods, however, in instances where recovery is claimed only over short periods, it could reflect a transitional period to nonviability, during which the cells can be rescued (Kell *et al.*, 1998). An explanation of how non-culturable cells arise that retain high levels of metabolic activity, acting as 'bags of enzymes' has recently been proposed. It is based on the fact that, stresses disturb cellular homeostasis, inhibiting growth, but not affecting metabolic activity. Therefore an imbalance occurs between anabolism and catabolism, producing a free radical burst, resulting in cell death. It was proposed that due to their higher catabolism, exponential phase cells are more sensitive than stationary phase cells (Dodd *et al.*, 1997).

Avoiding the use of words such as 'viability', 'live' and 'dead', in favour of precise terms describing the methods used e.g. plate counts, MPN count, proportion of dye-positive cells would minimise misinterpretation within this field (Kell *et al.*, 1998). If VBNC cells exist then formation should be an energy dependent process, and possess a definable phenotype in morphological or physiological terms by microscopy, flow cytometry or mapping of gene expression (Barer *et al.*, 1993 and Kell *et al.*, 1998).

1.2.8.3 Methods of detecting cellular activity in the non-culturable state

Bacterial activity can be determined in populations or single cells. Bulk assays require the entire population to be non-culturable when assigning properties, otherwise any derived correlation between activity and culturability will be obscured by the presence of other physiological types (Kell *et al.*, 1998). Many different assays have been developed, and their merits shall be discussed below. Structures of those compounds used in viability staining studies are shown in Figure 1.3.

1.2.8.3.1 Direct viable count (DVC)

This method was developed by Kogure *et al.*, 1979, for enumerating metabolically active unculturable marine bacteria. Small amounts of nutrient (yeast extract), and nalidixic acid (DNA gyrase inhibitor) are added to cells for 6 hours. Nalidixic acid prevents septation due to its tight coupling to DNA synthesis, therefore cells that retain the ability to utilise nutrients (substrate responsive) elongate. This technique detects metabolic activity in many Gram-negative species that enter the 'ABNC' state including *C. jejuni* (Rollins & Colwell, 1986). Recently the DVC technique has been modified, using other DNA gyrase inhibitors that are inhibitory to Gram-positive bacteria, such as, ciprofloxacin and norfloxacin allowing identification of the 'ABNC' state in organisms resistant to nalidixic acid (Servis *et al.*, 1995 and Barcina *et al.*, 1997). However, the validity of the DVC assay has been questioned. It is known that quinolones induce the SOS response in *E. coli* resulting in filamentation, perhaps providing the basis of the DVC assay rather than substrate responsiveness (Barer *et al.*, 1993).

1.2.8.3.2 Respiratory activity: INT and CTC

The electron accepting tetrazolium salts (INT and CTC) are reduced by active dehydrogenase enzymes to their respective formazan compounds. In the case of INT the precipitate is an intracellular, optically dense, red inclusion, visualised by bright-field microscopy, whilst CTC-formazan is a red fluorescent deposit, and can be visualised by fluorescent microscopy (Thom *et al.*, 1993). INT and CTC reduction is coupled to respiratory electron transport. In *E. coli*, INT and CTC were reduced by NADH dehydrogenase and succinate dehydrogenase. INT was also reduced by ubiquinone and possibly cytochromes b_{555} and b_{556} and CTC by the terminal oxidases (Smith & McFeters, 1997).

INT counter-stained with acridine orange determines bacterial respiratory activity in aquatic (Zimmerman *et al.*, 1978) and marine systems (Rodriguez *et al.*, 1992). Plate counts correlated with INT-stained cell counts for bacterial cultures (Betts *et al.*, 1989), demonstrating that INT accumulation indicates cell viability. In contrast, CTC may have toxic effects on bacterial metabolism, therefore underestimating the number of actively respiring cells, due to disruption of normal metabolic function, probably by energy depletion (Ullrich *et al.*, 1996). Therefore, in studies using tetrazolium salts, the possible underestimation of active cells should be noted. However, many other groups, found that active and total counts correlated, using similar CTC concentrations (Rodriguez *et al.*, 1992 and Boucher *et al.*, 1994). The use of INT and CTC in reducing environments leads to non-biological reduction, perhaps explaining the observations that INT is reduced by non-living detritus and exopolymers (Smith & McFeters, 1997).

1.2.8.3.3 Fluorochromes: acridine orange and DAPI

Acridine orange (AO) is a non-specific fluorochrome which binds to cell components as well as DNA and RNA (Chen & Koopman, 1997). Acridine orange direct counts (AODC) have been proposed as a means of distinguishing live and dead cells. Acridine orange fluoresces orange when interacting with RNA and green with DNA. Orange cells are presumed to be viable due to the high levels of RNA present within the cell. However, colour is dependent on pH, incubation time, AO concentration, growth medium and growth phase (Oliver, 1993). Acridine orange is not a good indicator of the metabolic status of the cell, and is mainly used for total counts (Oliver, 1993). Despite some published material using AO as an indicator of metabolic activity, such cells **will not** be termed 'ABNC', because other physical factors influence the fluorescence.

DAPI is non-intercalating, polar, and specific for the minor groove of DNA, showing a particular tropism for AT-rich sequences (Kapuscinski & Szer, 1979), exhibiting blue fluorescence upon excitation at 365 nm. Fluorescence is stable over a wide pH range and is independent of temperature (Chen & Koopman, 1997). It is mainly used as a counterstain to 'activity' dyes, allowing total and 'ABNC' counts to be performed on the same sample (Rodriguez *et al.*, 1992).

1.2.8.3.4 Propidium iodide and ethidium bromide

The nucleic acid intercalators, propidium iodide (PI) and ethidium bromide (EtBr) bind to both DNA and RNA (Davey & Kell, 1996). PI is more water-soluble and less membrane permeant. Once excited at a wavelength of 617 nm (PI), and 605 nm (EtBr), the dyes fluoresce red (Davey & Kell, 1996, and Haughland, 1999). Both dyes are excluded from viable cells with intact cytoplasmic membranes, and have

been utilised as nucleic acid markers in double-staining strategies with membrane potential dyes to study stressed *E. coli* and *S. typhimurium* populations via flow cytometry (López-Amorós *et al.*, 1995).

1.2.8.3.5 Homeostatic stability dyes: rhodamine 123 and oxonol

Retention of membrane potential, demonstrates cellular homeostasis, and a functional cell membrane. Two membrane potential dyes are being used with increasing regularity, rhodamine 123, and oxonol. These two dyes differ with respect to the metabolic status of the cells stained.

Rhodamine 123 (Rh123) is a cationic, lipophilic dye, accumulated cytosolically by cells with an intracellular negative transmembrane electrochemical potential (Mason *et al.*, 1995), equilibrating across the membrane and emitting green fluorescence (Davey & Kell, 1996). Rh123 binds intracellularly to lipid compounds. Whilst the free dye concentration is below the saturation point for the available binding sites, accumulation is membrane potential-dependent. Ionophores that dissipate membrane potential, such as CCCP prevent Rh123 uptake (Nebe-Von Caron & Badley, 1995).

Rh123 fluorescence correlates with plate counts of *E. coli* and *S. typhimurium* cells stressed in a marine microcosm (López-Amorós *et al.*, 1995). Conversely in another study, Rh123 only exhibited small changes in fluorescence between viable and non-viable bacterial populations (Mason *et al.*, 1995). Flow cytometry with Rh123 has been used to study heterogeneous populations of *Micrococcus luteus* (Kaprelyants & Kell, 1992), *Klebsiella pneumoniae* (Diaper & Edwards, 1994), and *Pasteurella piscicida* (Margariños *et al.*, 1997). Furthermore, it was possible to categorise a heterogeneous starved *M. luteus* population into culturable, non-viable

(dead), and metabolically active, non-culturable cells (Kaprelyants & Kell, 1992). Apart from the lack of a 'sharp' distinction between viable and non-viable cells, Gram-negative bacteria require treatment with EDTA to permeabilise the outer membrane to allow the dye to accumulate within the cell (Mason *et al.*, 1995). This is an invasive process and should be taken into account when proposing an 'ABNC' population via this method.

Oxonols are lipophilic anionic dyes that accumulate in organisms with an intracellular positive transmembrane electrochemical potential, binding to the outer lipoprotein layer of the cytoplasmic membrane, resulting in a red spectral shift. Therefore, the fluorescent response is the inverse of that exhibited by Rh123. Oxonol exhibits a large change in fluorescence between culturable and non-viable *E. coli, S. typhimurium*, and *S. aureus* cells stressed by heat and gramicidin treatment. Oxonol was proposed to be a more useful viability dye than Rh123 because the fluorescent response was more definitive (Mason *et al.*, 1995 and Jepras *et al.*, 1995). Oxonol has been used successfully with flow cytometry, for stressed *Aeromonas salmonicida* (Deere *et al.*, 1995), *E. coli*, and *S. typhimurium* cells (López-Amorós, *et al.*, 1995). An advantage of the oxonol dyes is their ability to diffuse across both Gram-positive and Gram-negative cell walls, therefore it is not as invasive to Gram-negative bacteria as treatment with rhodamine 123 (Haughland, 1999).

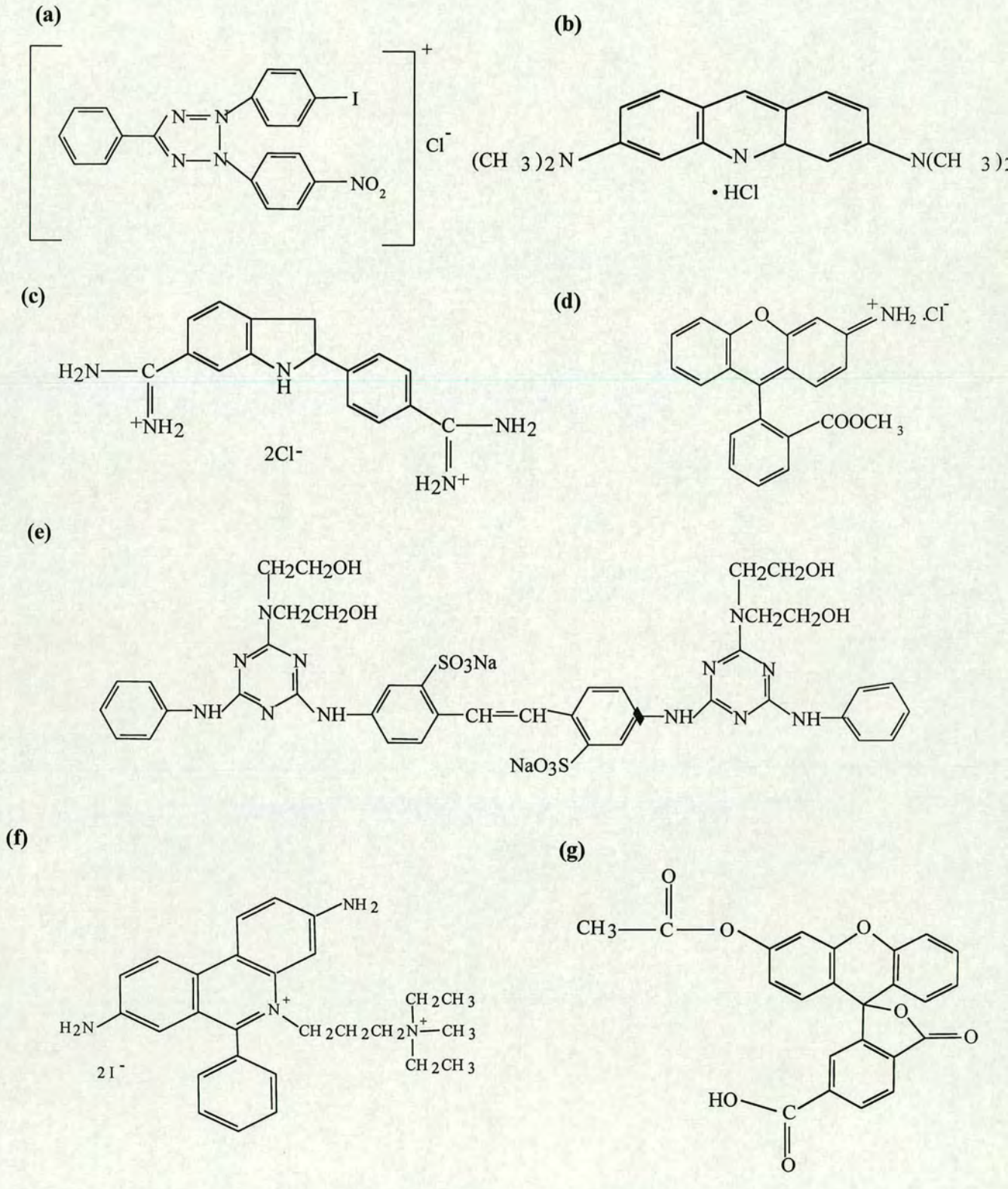

Figure 1.3: Chemical structures of compounds used in this study. (a) INT, (b) acridine orange, (c) DAPI, (d) rhodamine 123, (e) calcofluor white, (f) propidium iodide and (g) 6-carboxyfluorescein diacetate

1.2.8.3.6 Fluorescent brighteners: calcofluor white

Calcofluor white M2R (CFW; fluorescent brightener 28) is one of a group of stains known as fluorescent brighteners. CFW fluoresces bright blue when excited under UV illumination (Mason *et al.*, 1995). CFW has no bactericidal action and permits bacterial division (Darken, 1962), binding strongly to β(1-3) and β(1-4)-linked polymers, including cellulose and chitin (Maeda & Ishida, 1967 and Berglund *et al.*, 1987). CFW has been used to stain fungal cell walls (Thrane *et al.*, 1999), mammalian cells, diatoms, plant protoplasts (Berglund *et al.*, 1987), determine bacterial viability (Mason *et al.*, 1995) and to differentiate between microbial species (Davey & Kell, 1997).

Viable cells exclude the dye, but non-viable cells take up the dye, appearing brightly fluorescent (Fritz & Treimer, 1985 and Mason *et al.*, 1995). CFW fluorescence correlates with PI-stained cell counts, and inversely with culturability, and FDA-stained cell counts. The mode of binding is unknown although it has been proposed that CFW is excluded by living cells that retain a negative membrane potential (Mason *et al.*, 1995). CFW has been used to study stressed *E. coli*, *S. typhimurium*, and *S. aureus* cells with flow cytometry, where a good distinction between live and dead cells was obtained, comparable to oxonol, and better than rhodamine 123 (Mason *et al.*, 1995).

1.2.8.3.7 Fluorogenic substrates: carboxyfluorescein

Fluorogenic substrates are lipophilic, uncharged, non-fluorescent compounds, that readily diffuse across cell membranes. Uptake by viable cells promotes hydrolysis by non-specific esterases to polar fluorescent compounds that are retained by cells

with intact membranes, whilst cells with dysfunctional membranes rapidly lose the dye (Jepras *et al.*, 1995). The necessity for esterases means these dyes are only of use in microbial species that contain such enzymes (Diaper & Edwards, 1994). At high pHs (>7.6) CFDA and other FDA derivatives autohydrolyse irrespective of the presence of cells (Haughland, 1999). CFDA has been used as a viability probe in bacteria such as *Ps. aeruginosa*, *E. coli* (Jepras *et al.*, 1995), *Legionella pneumophila* (Yamamoto *et al.*, 1996), *Listeria monocytogenes* (Jacobsen *et al.*, 1997), and *Klebsiella pneumoniae* (Diaper & Edwards, 1994). In a comparative study of fluorogenic substrates no single dye was universal in staining all bacterial species tested, perhaps due to lack of esterase activity or accessibility to the dye (Diaper & Edwards, 1994).

1.2.8.3.8 Alternative methods of detecting activity in non-culturable cells

The majority of studies on non-culturable cells use metabolic stains as the basis of designating activity to non-culturable cells, however the past decade has seen the emergence of other methods of detecting cellular activity. Uptake of tritiated thymidine has been detected in non-culturable *H. pylori* cells (Shahamat *et al.*, 1993), and β-galactosidase activity has been demonstrated in non-culturable enterobacteria, indicating the potential of non-culturable cells to influence the environment (Nwoguh *et al.*, 1995).

Reporter genes such as the green fluorescent protein (*gfp*) and luciferase (*lux*) have gained prominence as viability determinants as they indicate transcriptional and translational capacity of the cell. *S typhi gfp*-tagged 'ABNC' counts correlated better with plate counts than *lux*-tagged cells (Cho & Kim, 1999).

1.2.8.4 Resuscitation from the non-culturable state in non-campylobacters

The only definitive way of proving whether non-culturable cells are actually alive is by demonstration that the cells revert to the plateable state (Kell *et al.*, 1998). However, the literature is inconclusive with studies being incomparable (species and stress conditions vary) and the use of both *in vivo* and *in vitro* resuscitation models making interpretation difficult (Barer *et al.*, 1993). In addition, there exists the problem of confirming that observed 'resuscitation' is not the result of a few conventionally plateable cells (Ravel *et al.*, 1995 and Dukan *et al.*, 1997). The problem of residual culturable cells can be overcome by sufficient dilution of the inoculum (Oliver & Bockian, 1995), however this has added problems in animal models because the infectious dose of 'ABNC' cells is unknown. Resuscitation may be a temporal process as observed in the *V. cholerae* (Wai *et al.*, 1996 and Colwell *et al.*, 1996), and *S. enteritidis* (Roszak *et al.*, 1984) two-stage process of non-culturability. In a review by Kell *et al.*, 1998, three cases were considered to demonstrate successful reversal of non-culturable cells. These were the resuscitation of *M. luteus* in the presence of a factor produced by viable bacteria (Kaprelyants, *et al.*, 1994), conversion of non-culturable *V. cholerae* cells by heat shock (Wai *et al.*, 1996), and resuscitation of *C. jejuni* cells 23-fold under MPN conditions (Bovill & Mackey, 1997).

Resuscitation may require specific environmental factors supplied by autochthonous organisms within the aquatic system. For example, recovery of non-culturable *L. pneumophila* cells has been reported in *Acanthamoeba castellanii* (Steinert *et al.*, 1997), but not in the ciliated protozoan, *Tetrahymena pyriformis* (Yamamoto *et al.*, 1996). Alternatively, the host organism may supply the stimulus

for resuscitation, as has been observed in *V. vulnificus* upon passage through the mouse model (Oliver & Bockian, 1995), clams and murine macrophage cultures (Oliver, 1993). It is possible that specific chemicals trigger reversion to the culturable state. Osmotically stressed *E. coli* cells were resuscitated by the addition of betaine, in the presence of chloramphenicol that inhibits protein synthesis and cell division (Roth *et al.*, 1988). Resuscitation of cold-shocked, non-culturable *V. cholerae* cells was prompted by heating the cultures for 1 min at 45°C. Over 1000 colonies were recovered, reliant on the presence of NH_4Cl (Wai *et al.*, 1996).

In certain cases, for example, *V. vulnificus* (Nilsson *et al.*, 1991) or *V. cholerae* cells (Wai *et al.*, 1996), temperature upshift is the trigger for resuscitation. Resuscitated cells increase in size, becoming rod-shaped, and are reliant on protein and peptidoglycan synthesis (Nilsson *et al.*, 1991). Conversely, resuscitation of non-culturable cells could not be achieved by any combination of temperature and nutrient upshifts, and was attributed to outgrowth of injured or culturable cells (Weichart *et al.*, 1992 and Weichart & Kjelleberg, 1996), indicating the difficulties in interpretation of resuscitation data (Kell *et al.*, 1998). *In vitro* evidence for resuscitation of *V. vulnificus* non-culturable cells involved diluting the cells by 10^3-fold eliminating the possibility of residual culturable cells being present. Upon temperature upshift the cells regained culturability to their original total counts (Whitesides & Oliver, 1997). Further evidence for 'true' resuscitation arose from the observation that 8h were required for culturable forms to appear, consisting of a 7h lag, and 1h resuscitation period. A generation time of 6 mins would be required if division of culturable cells was the reason for the observed increase in culturability (Whitesides & Oliver, 1997).

The non-culturable coccoid form of *H. pylori* has been proposed as a dormant cell aiding survival in harsh conditions (Bode *et al.*, 1993 and Benaïssa *et al.*, 1996). Published reports on resuscitation from the coccoid state in *H. pylori*, highlights the contradictory nature of this field. Colonisation of BALB/c mice occurred using both spiral and aged coccoid cells. In all colonised mice, a systemic antibody response to *H. pylori* was observed (Cellini *et al.*, 1994 and Wang *et al.*, 1997), however, optical density was used to assess the number of bacteria inoculated, hence the possibility of residual, culturable spirals being present exists (Kusters *et al.*, 1997). Conversely, coccoid cells did not colonise gnotobiotic piglets (Eaton *et al.*, 1994), or chick eggs (Enroth & Engstrand, 1996). Contradictory *in vitro* reports exist, with reversion of starved, coccoid cells to the spiral state being demonstrated microscopically, upon incubation in fresh medium (Anderson *et al.*, 1997), whilst a study utilising a variety of resuscitation methods (sonication, heat-shock, and acid-shock) on 45d old, cold-incubated coccoid cells proved unsuccessful (Cellini *et al.*, 1998). It is possible that the observation of resuscitation in the study of Andersen, *et al.*, 1997, was due to a few spiral cells, swimming into the field of view. The contradictory nature of these reports may be explained by a lack of uniformity between the methods of obtaining coccoid cells and resuscitation. There are believed to be two types of coccoid cells in *H. pylori*, viable and non-viable, dictated by induction conditions, those formed in the presence of antibiotics were proposed to be 'alive', whilst those formed under starvation were assumed to be 'dead' on the basis of structural integrity (Nilius *et al.*, 1993). However, the general consensus is that they are the 'morphologic manifestation of cell death'. Certainly, starved coccoid cells lost membrane potential

and were reduced in nucleic acid content compared to unstarved spiral cells (Kusters *et al.*, 1997).

The need to eliminate the possibility of residual culturable cells is exemplified by some work on nonculturable *V. cholerae* cells (Ravel *et al.*, 1995). A temperature upshift from 4 to 30°C for 72h, resulted in an increase in plate counts. However, it was concluded that outgrowth of a few residual culturable cells resulted in the observed 'resuscitation', (Ravel *et al.*, 1995). Likewise, non-culturable *E. coli* cells formed by HOCl treatment showed increases in the numbers of culturable cells upon stress removal, primarily due to regrowth of culturable cells. However, the growth rate was higher than expected, and it was concluded that some reversal of non-culturable cells was also occurring (Dukan *et al.*, 1997). Such problems in eliminating culturable cells as the cause of resuscitation, highlight the difficulties encountered within this field (Kell *et al.*, 1998).

1.2.8.5 The coccoid state (dormant or dead?)

Stresses, such as, high and low temperature, starvation and oxidative shock, cause campylobacters to morphologically transform from the plateable spiral state to the non-plateable coccoid state (Figure 1.4), designated a 'VBNC' state (Rollins & Colwell, 1986). The recent demonstration that coccoid cells show higher resistance to chlorine based disinfectants than culturable spiral cells is a worrying proposition, highlighting the necessity for research into this state (Rowe *et al.*, 1998).

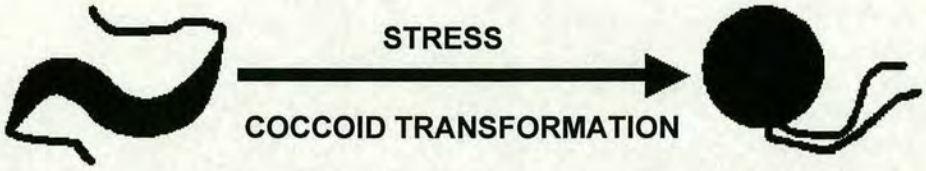

Figure 1.4: Diagrammatical representation of the present hypothesis of coccoid transformation as proposed by Rollins & Colwell, 1986

Light, hydrogen peroxide, and superoxide anions, are implicated as agents that cause rapid transformation upon oxygenation of campylobacters (Moran & Upton, 1987a,b). In a comparative study using continuous culture, only high oxygen tension in conjunction with carbon limitation resulted in coccoid formation. Low or high pH, nutrient limitation (iron, carbon, or phosphate) or high oxygen tension alone never induced the coccoid state (Harvey & Leach, 1998). Most *Campylobacter* and *Helicobacter* species enter the coccoid state, particularly in ageing cultures, although ease of coccoid transformation is species dependent (Tables 1.1 and 1.2), with the thermophilic campylobacters entering this state very rapidly, whilst *C. fetus* requires prolonged periods of time (Ogg, 1962 and Karmali *et al.*, 1981). Coccoid cells are also observed in other stressed bacteria, such as *Vibrio* spp. (Hallock, 1959), *Photobacterium damselae* (Fouz *et al.*, 1998) and *Yersinia ruckeri* (Romalde *et al.*, 1994).

1.2.8.5.1 Structure of coccoid cells

S-shaped spirals are 2.1-3.0 μm long and 0.26-0.7 μm wide (Pead, 1979, Merrell *et al.*, 1981, and Moran & Upton, 1987b), whereas coccoid cells are smaller, averaging 0.2-1.2 μm in diameter (Pead, 1979, Koike & Shimazaki, 1982 and Moran & Upton, 1987b). Coccoid cells are heterogeneous in diameter falling into two populations, small (0.2-0.4 μm) and large (>0.6 μm), on the evidence of filtering

(Koike & Shimazaki, 1982). Aged cultures, though predominantly coccoid, are heterogeneous making fractionation and purification of coccoid forms elusive (Marsh *et al.*, 1969). Electron and light microscopy studies indicate morphological differences between spiral and coccoid cells.

1. **Spirals**: The cytoplasm is closely attached to the cell wall (Ng *et al.*, 1985), with most spirals possessing bipolar flagella, attached to a concave depression (Pead, 1979, and Brock & Murray, 1987). In older, non-culturable populations formed at 4°C, spirals are still present, though they appear degenerate due to protoplast shrinkage (Jones, *et al.*, 1991).

2. **Coccoid**: The cytoplasm is detached from the cell wall (Ng *et al.*, 1985), whilst the outer membrane is enlarged in relation to the rest of the coccoid cell, but is thinner than the outer membrane of spirals (Moran & Upton, 1987b). There is loss of cell integrity and blebbing in some coccoid cells (Buck *et al.*, 1983, Moran & Upton, 1987b, and Thomas *et al.*, 1999b), however the majority maintain an intact membrane structure, though occasionally spheroblasts or 'ghost cells' are observed (Rollins & Colwell, 1986, and Jacob *et al.*, 1993). Up to 20% of cells formed at 4°C in pond water were coccoid, most showing degenerate features, but 6% had an intact cell wall and appeared viable (Jones *et al.*, 1991a). No nucleoplasm can be observed, and relatively few ribosomes (Brock & Murray, 1987). Coccoid cells retain the normal length flagella (Moran & Upton, 1987b, and Jones *et al.*, 1991b), forming a bundle in the coccoid cell (Jones *et al.*, 1991b). Coccoid cells are extremely 'sticky' (Brock & Murray, 1987), forming large, irregular clumps (Rollins *et al.*, 1983, Rollins &

Colwell, 1986, and Boucher *et al.*, 1994), enmeshed in amorphous material (Rollins *et al.*, 1983).

The presence of extracellular debris in preparations of coccoid cells (Buck *et al.*, 1983, and Moran & Upton, 1987b) indicates leakage of cytoplasmic contents during coccoid transformation, such as DNA, RNA (Moran & Upton, 1986), and ATP (Beumer *et al.*, 1992, and Hazeleger *et al.*, 1995). Coccoid cells formed at higher temperatures (25-42°C) contain less nucleic acids, compared to those formed at low temperatures (4°C), where levels were comparable to spirals (Hazeleger *et al.*, 1994). ATP leakage was dramatic in coccoid cells formed at 20-25°C (Beumer *et al.*, 1992, and Hazeleger *et al.*, 1995), whereas coccoid cells formed at 4-12°C maintained intracellular ATP levels better, indicating membrane integrity (Hazeleger *et al.*, 1995). Energy remains potentially available for initiation of growth in coccoid cells, particularly those formed at 4-12°C (Hazeleger *et al.*, 1995).

Coccoid cells were more resistant to lysis with detergents and lysozyme than spirals (Moran & Upton, 1986), suggesting fundamental differences between the two forms. The peptidoglycan (PG) yield of coccoid cells is lower than spirals, suggesting resistance to lysis, as both spiral and coccoid cultures possessed identical PG components and composition. The authors concluded that the transformation process is probably due to enzymatic degradation of PG (Amano & Shibata, 1992). The concentration of the polyamine, spermidine increased in early log phase, indicating a role in nucleic acid and protein synthesis. It is present in spiral rather than coccoid cells. Cadaverine content increased concomitant with a decrease in coccoid forms. Interestingly, cadaverine is essential for cell surface integrity (Suzuki *et al.*, 1994), and it has been shown that many coccoid cell appear degenerate (Buck *et al.*, 1983).

The membrane fatty acid profiles of coccoid cells formed at 4-12°C were similar to spirals, hence *C. jejuni* does not adapt the fatty acid content of the membrane during transition to the coccoid form at low temperatures (Hazeleger *et al.*, 1995), whilst *C. coli* cells incubated at low temperatures show an increase in long chain and cyclopropyl derivatives, aiding membrane fluidity (Höller *et al.*, 1998). It is unusual that *C. jejuni* coccoid cells formed at low temperatures contained unchanged amounts of short chain fatty acids, because in order to maintain membrane fluidity short-chain fatty acids decrease, as observed in *V. vulnificus* and *A. salmonicida* 'ABNC' cells (Oliver, 1993).

1.2.8.5.2 Activity of coccoid cells

Coccoid formation occurs in the presence of protein synthesis inhibitors and γ-irradiation, hence *de novo* protein synthesis is not required for coccoid transformation, and is a passive process (Hazeleger *et al.*, 1995 and Thomas *et al.*, 1999b), adding weight to the hypothesis that coccoid forms are degenerate (Boucher *et al.*, 1994). No temperature dependent pattern of protein synthesis could be detected in cold-shocked *C. coli* cells (Jacob *et al.*, 1993 and Höller *et al.*, 1998), and lipooligosaccharide patterns were unaltered (Jacob *et al.*, 1993). Aged, coccoid *Campylobacter* cultures had decreased amounts of 28 and 33 kDa proteins compared to spiral cultures (Shibata *et al.*, 1991).

Non-culturable, coccoid *C. jejuni* cells possess dehydrogenase activity, as indicated by the reduction of tetrazolium salts, INT (Boucher *et al.*, 1994), and CTC (Boucher *et al.*, 1994, Bovill and Mackey, 1997 and Cappelier *et al.*, 1997). The necessary incubation time with the tetrazolium salt increased as cultures turned coccoid (Boucher *et al.*, 1994), perhaps indicating gradual shut-down of respiratory

activity, as the cells enter a dormant state. Respiration as monitored by glutamate utilisation was greater at 37°C than at 27°C. It was proposed that at higher temperatures (20-30°C), any substrates that are present will be utilised rapidly leading to a more rapid decline in culturability (Rollins & Colwell, 1986). Excretion of organic acids indicates metabolic activity. At growth permissive temperatures (37°C), *C. coli* produces a characteristic profile of organic acids (succinate, lactate and acetate) with a 4h lag period (Höller *et al.*, 1998). A temperature downshift (4-20°C) altered this pattern, producing a lag of 7h, and lower succinate, lactate and acetate levels, even after entry into the nonculturable state (Höller *et al.*, 1998).

In conclusion, there are many different phenotypes of coccoid cells, and their characteristics are probably determined by the conditions under which they are formed. Coccoid cells formed at low temperatures (4°C) have characteristics comparable to spirals (Hazeleger *et al.*, 1995), however, the majority of evidence indicates that the coccoid state is degenerate (Koenraad *et al.*, 1997).

1.2.8.6 Non-culturable spiral Campylobacters

Recently it has been demonstrated by various groups, that cells incubated at 4°C, are predominantly spiral in morphology (Fearnley *et al.*, 1996 and Federghi *et al.*, 1998). In microcosm water held at 4°C under aerobic atmosphere, it was noted that entry into the ABNC state was strain-dependent with only 3 of 36 strains showing CTC-reducing activity in the non-culturable state. The notable feature of these three strains was that they retained spiral morphology (Federghi *et al.*, 1998), displaying a progressive increase in cell volume (Tholozan *et al.*, 1999). Both the membrane potential and internal potassium content were lower than in culturable cells. The decrease in internal K^+ levels and increase in cell volume probably results from

exposure to the hypoosmotic environment, and subsequent cellular adaptation (Tholozan *et al.*, 1999). Non-culturable spirals formed at 4°C possess intact DNA over extended incubation periods (Lázaro *et al.*, 1999). The ATP and ADP contents of ABNC cells gradually decreased to undetectable levels, whilst AMP levels were maintained, although at decreased levels. These perhaps minimise cell maintenance requirements (Tholozan *et al.*, 1999). The discovery of this non-culturable state further complicates the question of whether non-culturable *C. jejuni* cells are a health hazard.

1.2.8.7 Resuscitation in non-culturable Campylobacters

Unequivocal evidence of the viability of the coccoid, non-culturable state would be provided if such cells could be resuscitated. Conflicting evidence exists concerning *C. jejuni* resuscitation studies. Non-culturable *C. jejuni* cells formed at 4°C are reported to colonise mice (Jones *et al.*, 1991 and Cappelier *et al.*, 1999a), chick models (Stern *et al.*, 1994 and Cappelier *et al.*, 1999a) and egg passage (Cappelier *et al.*, 1999b). Passage of non-culturable *C. jejuni* through a rat intestine resulted in resuscitation to culturable, fully virulent cells (Saha *et al.*, 1991). Conversely, lack of colonisation of chicks has been reported with non-culturable *C. jejuni* cells formed at 4°C (Fearnley *et al.*, 1996, and Van de Giessen *et al.*, 1996), 37°C (Medema *et al.*, 1992), and in mice, rabbits and human volunteers at 20°C (Beumer *et al.*, 1992). It has been reported that microcosm incubated cells lose infectivity before culturability (Fearnley *et al.*, 1996), however this has been reported to be regained upon animal and egg passage (Cappelier *et al.*, 1999a+b). This confusion, may be due to use of selective media to determine non-culturability in those studies where resuscitation occurred (Jones *et al.*, 1991, and Stern *et al.*, 1994),

thus preventing detection of injured cells, that may be capable of initiating infection. Also the methods for creating non-culturable forms differ. One study with a lack of resuscitation used coccoid cells formed at 25°C (Medema *et al.*, 1992), proposed to be degenerate (Hazeleger *et al.*, 1995), whilst studies using cells incubated at 4°C, demonstrated degrees of resuscitation (Jones *et al.*, 1991, Stern *et al.*, 1994 and Cappelier *et al.*, 1999a). Such non-culturable, coccoid forms generally resemble spirals (Hazeleger *et al.*, 1995), and furthermore the non-culturable spiral form would be present (Cappelier *et al.*, 1999a). The differences observed in resuscitation studies using microcosms incubated at 4°C could be attributed to strain differences. In one study, only 3 out of 36 strains entered the ABNC state (Cappelier & Federghi, 1998), and were shown to resuscitate (Cappelier *et al.*, 1999a+b).

Two *in vitro* studies have been recently published. An increase in culturable, spiral cells occurred upon sparging aged, coccoid cultures with a microaerobic gas mixture. It was concluded that multiplication of residual culturable cells was the major factor contributing to this increase, although resuscitation of latent cells was not discounted (Bovill & Mackey, 1997). In systems incubated at 4°C that were thought not to support growth, transient increases in plate counts were observed in populations suspended in faecal emulsions and nutrient broth, but not in nutrient-free systems. These increases were attributed to transitions between the non-culturable and culturable states, as often the increases were too great to be accounted for by the growth of culturable cells (Ekweozor *et al.*, 1998).

Based on the above evidence, it appears that the coccoid, non-culturable state of *C. jejuni* is degenerate. However, the viability of the coccoid cell may depend upon the physiological condition under which they were formed (Hazeleger *et al.*, 1995).

The recent discovery of the non-culturable spiral state complicates the issue and may well be the true 'VBNC' state in campylobacters.

1.3 Aims of the thesis

The aims of the project were three-fold:

- to determine the singular effect of cold storage on the survival of *C. jejuni* in the plateable and non-plateable states, via a combination of plate counts, metabolic stains, resuscitation procedures and two-dimensional polyacrylamide electrophoresis (2D-PAGE).
- to characterise the combinatorial effects of cold-shock and other factors important to the maintenance of *C. jejuni* in the food industry, such as aerobic incubation, growth phase and disinfection processes such as, hydrogen peroxide, UV irradiation and trisodium phosphate.
- to determine the capacity of *C. jejuni* under the above stresses to induce stress responses with the production of specific stress proteins.

Section 2

Materials and Methods

2.0 Materials and Methods

2.1 Materials and chemicals

Chemicals and reagents

All chemicals were analytical grade, and purchased from the following companies:

Aldrich Chemical Company Ltd., New Road, Gillingham, Dorset,

Calbiochem-Novabiochem Ltd., Beeston, Nottingham,

Difco Laboratories Ltd., West Molesey, Surrey

Fluka Chemicals, New Road, Gillingham, Dorset,

Oxoid Ltd., Basingstoke, Hampshire, and

Sigma Chemical Company, Fancy Road, Poole, Dorset,

Nucleases and other enzymes

All nucleases (DNase/RNase) were obtained from:

Boehringer Mannheim, Bell Lane, Lewes, East Sussex,

Promega Corporation, Madison, Wisconsin, USA

New England BioLabs Ltd., Wilbury Way, Hitchin, Hertfordshire,

Radioisotopes

Trans^{35}S-label™ (1.4 mCi) possessing L-[^{35}S]-methionine (70%) and L-[^{35}S]-cysteine (15%), used for metabolic labelling was supplied by ICN Biomedicals Ltd., Wenman Road, Thame, Oxfordshire.

Autoradiography

Pre-flashed X-ray film was purchased from Agfa Gavaert N.V., B-2640, Mortsel, Belgium, whilst autoradiography cassettes were from Dupont Cronex®, either the Quanta-III DE (small format) or the Lightning-Plus EC (large format).

2D-PAGE and 1D-PAGE reagents and consumables

Immobiline® DryStrips (pH 3-10 L, 11 cm; IEF dimension), IPG-buffer (3-10 L), and Plusone™ DryStrip Cover Fluid were purchased from Pharmacia Biotech AB, Uppsala, Sweden. Urea (high purity) was obtained from Boehringer Mannheim and Ultra Pure Protogel® (30% [w/v] acrylamide, 0.8% [w/v] bisacrylamide stock solution [37.5:1]) was purchased from National Diagnostics, Atalanta, Georgia, 30336, USA. Sequencing Grade Modified Trypsin was purchased from Promega Corporation.

Silver stain SDS-PAGE Standards Low Range (rabbit muscle phosphorylase b, bovine serum albumin, hen egg white ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, and hen egg white lysozyme with respective M_r values of 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa) were purchased from Bio-Rad Laboratories Ltd., Maylands Avenue, Hemel Hempstead, Hertfordshire. Two-dimensional PAGE markers (amyloglucosidase 89/ 70 kDa, pI 3.8; ovalbumin 45 kDa, pI 5.1; carbonic anhydrase 29 kDa, pI 7.0; myoglobin 17 kDa, pI 7.6) were purchased from Sigma Chemical Company. GelCode® Blue Stain Reagent was purchased from Pierce, 3747, North Meridian Road, Rockford, IL 61105, USA.

Protran® nitrocellulose membrane was supplied by Schleicher & Schuell GmbH, Postfach 4, D-37582, Dassel, Germany, whilst the Enhanced Autoradiography system was purchased from EABiotech Ltd., Strathclyde Business Centre, Hamilton Road, Flemington. Glogos™ II Autorad Markers were purchased from Stratagene Ltd., Cambridge Life Sciences Park, Milton Road, Cambridge.

Membrane filters and microconcentrators

Membrane filters (25 mm diameter, 0.22 or 0.45 μm pore size) and ultrafiltration membranes (YM10; 62 mm diameter; 10 kDa pore size) were purchased from Millipore Corporation, Bedford, MA 01730, USA. Microconcentrators/filtrators (Centricon-3® = 3 kDa cut-off, and Vectaspin™ 3 = 10 kDa cut-off) were respectively supplied by: Amicon Inc., Beverly, MA 01915, USA and Whatman International Ltd., Whatman House, St Leonard's Road, Maidstone, Kent.

Microscopy consumables

Microscope slides and coverslips were supplied by Chance Propper Ltd., West Midlands, whilst lens-cleaning tissue was purchased from Whatman International Ltd., Kent. Immersion Oil (at 25°C, $n_e = 1.518$, and $n_D = 1.515$) was obtained from Zeiss, Germany.

2.2 Bacterial strains used in the study

Campylobacter jejuni 81116 (Penner type 6; NCTC 11828) was kindly donated by Dr D. Newell (Central Veterinary Laboratory, Surrey) and was used for most survival studies. Other *C. jejuni* strains used were obtained from Dr J. Moore (Queen's University of Belfast) and included NIC176, NIC35, NIC112, NIC45, and 2877. *Campylobacter coli* strains N43 and 11366 were studied as additional thermophilic campylobacters. Other bacterial species, used primarily in dye studies included, *Escherichia coli* B, *Salmonella typhimurium* LT5, *Klebsiella* spp, *Bacillus subtilis* W168, and *Staphylococcus aureus*. These were obtained from the microbiology teaching laboratory, University of Edinburgh.

2.3 Maintenance of bacterial strains

The cells were harvested in 50 mM phosphate/DMSO buffer (pH 7.0), consisting of 1.3 g $NaH_2PO_4.2H_2O$ and 2.3 g $Na_2HPO_4.2H_2O$ in 100 ml of a 15% (v/v) DMSO solution. The entire growth from a spread plate was suspended in 1 ml of phosphate/DMSO buffer (pH 7.0), and frozen at -80°C.

2.4 Preparation of *Campylobacter* media

Brucella-FBP media

Campylobacters were routinely cultured on Brucella-FBP agar plates. Brucella-FBP broth was used for most survival and resuscitation experiments. The agar plates and broths were prepared according to the manufacturers instructions (Table 2.1) from Brucella broth [Difco Laboratories] and used within two days. Stocks of FBP supplement (broth and plate strength) were prepared and stored at 4°C for up to 2 weeks without loss of toxic oxygen scavenging ability (Table 2.1), giving final concentrations of 0.9 mM iron (II) sulphate, 1.3 mM sodium metabisulphite, and

2.3 mM sodium pyruvate. For the purpose of selective media, 1% (w/v) deoxycholate or antibiotics giving final concentrations of 10 mg/l vancomycin, 5 mg/l trimethoprim and 2500 units/l polymyxin B, were added prior to pouring as required.

Constituent	Sterilise
Brucella broth: 28g Agar‡: 15g Distilled water: 1 litre	121°C for 20 min (15 psi) in autoclave **Final pH** = 7.0 ± 0.2 at 25°C
FBP supplement:	
(a) Broth strength:	0.025g each of iron (II) sulphate, sodium pyruvate and sodium metabisulphite per 5 ml of sterile water, prepared as a 50 ml stock. After filter sterilisation, 300 µl was added per 10 ml Brucella broth.
(b) Plate strength:	0.25 g of each component per 5 ml of sterile water, and prepared as a 50 ml stock. After filter sterilisation 5 ml was added to 500 ml Brucella agar.

Table 2.1: Composition of Brucella-FBP agar and Brucella-FBP broths. ‡ = absent from broth. Brucella broth [Difco Laboratories]: 10 g bacto peptamin, 10 g bacto tryptone, 1 g bacto dextrose, 2 g bacto yeast extract, 5 g NaCl, 0.1 g sodium metabisulphite per litre.

Chemically defined broths

Two defined broths (minus methionine and cysteine) were used to culture *C. jejuni* for metabolic labelling experiments: MEMeagles broth (Minimal Essential Medium; Sigma) and ACES buffered chemically defined (ABCD) broth (Pine *et al.*, 1987). Their compositions are detailed in Table 2.2, whilst modifications, supplements and preparation of the MEMeagles and ABCD broths are described in Tables 2.3 and 2.4 respectively. MEMeagles is a mammalian tissue culture broth based on MEMα, but deficient in a number of nutrients including, methionine and cysteine. Other essential minerals and vitamin were added as supplements to provide

final concentrations equivalent to those in MEMα. The full pulsing medium for *C. jejuni* is prepared as described in Table 2.3.

Component	Mol. wt (M_r)	MEMeagles		ABCD	
		Concentration (g/l)	Molar amount (μM)	Concentration (g/l)	Molar amount (μM)
ACES	182.2	-	-	10	54900
$CaCl_2.2H_2O$	147	0.26	1800	0.56	3800
$MgSO_4.7H_2O$	246.5	0.1	400	0.22	870
myo-inositol	180.2	0.002	10	0.002	20
Nicotinamide	122.1	0.001	8.0	0.001	8
D-pantothenoate	476.5	0.001	2.0	0.002	4
Thiamine.HCl	337.3	0.001	3.0	0.002	6
L-arginine	174.2	0.13	730	0.1	570
L-histidine	155.2	0.04	270	0.1	640
L-isoleucine	131.2	0.05	400	0.1	760
L-leucine	131.2	0.05	400	0.1	760
L-lysine.HCl	182.7	0.07	400	0.1	550
L-phenylalanine	165.2	0.03	200	0.1	610
L-threonine	119.1	0.05	400	0.1	840
L-tryptophan	204.2	0.01	50	0.1	500
L-tyrosine	181.2	0.05	290	0.05	280
L-valine	117.2	0.05	400	0.1	850
L-alanine	89.1	-	-	0.1	1122
L-asparagine.H_2O	150.1	-	-	0.1	666
L-aspartic acid	133.1	-	-	0.1	751
L-glutamine	146.2	-	-	0.1	684
L-glutamic acid	147.1	-	-	0.1	680
Glycine	75.1	-	-	0.1	1332
L-proline	115.1	-	-	0,1	869
L-serine	105.1	-	-	2.0	19030
L-cysteine	121.2	-	-	0.5	4125
L-methionine	149.2	-	-	0.1	670
Glutathione (reduced)	307.3	-	-	0.5	1627
D-Glucose	180.2	1.0	5549	-	-
KCl	74.6	0.4	5362	-	-
$NaHCO_3$	84.0	2.2	26190	-	-
KH_2PO_4	136.1	6.8	50000	-	-
NaH_2PO_4	120.0	0.12	1020	-	-
Folic acid	477.4	0.001	2.1	-	-
Riboflavin	376.4	0.0001	0.27	-	-
Pyridoxal.H_2O	203.6	0.001	5.0	-	-
Choline chloride	139.6	0.001	7.2	-	-
Phenol red	376.4	0.01	26.6	-	-
D-Biotin	244.3	-	-	0.0001	0.4
DL-Thioctic acid	206.3	-	-	0.0001	0.5
Coenzyme A	820.4	-	-	0.0001	0.12
Haemin	652	-	-	0.002	3
α-ketoglutarate	168.1	-	-	1.0	6000
Sodium pyruvate	110	-	-	1.0	9100
$FeSO_4.7H_2O$	278	-	-	0.04	144
$MnCl_2$	125.8	-	-	0.02	159
NH_4VO_3	117	-	-	1.17	10000
Na_2SO_4	142	-	-	0.15	1056
$ZnSO_4.7H_2O$	287.5	-	-	0.02875	100
$CoCl_2$	129.8	-	-	0.476	3667
$NiSO_4.6H_2O$	262.9	-	-	0.526	2000
$CuSO_4.5H_2O$	249.7	-	-	0.025	210
$NaMO_4$	206	-	-	1.21	5874

Table 2.2: Chemical composition of MEMeagles broth (Sigma) and ABCD broth (Pine *et al.*, 1987)

Supplement	Composition	
1. Nutrient supplement: (×100 concentration)	L-alanine	0.25 g
	L-asparagine	0.25 g
	L-aspartic acid	0.3 g
	L-glutamine	2.92 g
	L-glutamic acid	0.75 g
	Glycine	0.5 g
	L-proline	0.4 g
	L-serine	0.25 g
	L-ascorbic acid	0.5 g
	make up to 100 ml with distilled water, dispense into 20 ml aliquots, and filter sterilise for purity. Store at 4°C.	
2. Biotin supplement: (×1000 concentration)	10 mg biotin dissolved in 10 ml distilled water. Filter sterilise for purity. Store at 4°C.	
3. FBP supplement †:	Iron (II) sulphate	2 mg
	Sodium metabisulphite	1 g
	Sodium pyruvate	1 g
	make up to 100 ml with distilled water, dispense into 20 ml aliquots, and filter sterilise for purity. Store at 4°C.	
Composition per 25 ml:	MEMeagles broth	23.725 ml
	Nutrient supplement	0.5 ml
	Biotin supplement	2.5 µl
	FBP supplement	0.75 ml

Table 2.3: Preparation of MEMeagles broth and the constituent supplements. † = Final concentration of FBP constituents are: 2 µm iron (II) sulphate, 1.3 mM sodium metabisulphite, and 2.3 mM sodium pyruvate. Note that the iron concentration was reduced (2 µm compared to 900 µm in Brucella-FBP broth) due to formation of an inhibitory precipitate when the higher concentration was used.

ABCD broth was prepared according to instructions (Pine *et al.*, 1987; Table 2.4). Omission of haemin did not affect the growth curve or morphology of the cells. Methionine and cysteine were omitted in order to achieve clean pulse-labelling during the 2D-PAGE experiments.

Solution	Composition	
Solution 1:	ACES buffer	10 g
(per 500 ml of distilled water)	KH_2PO_4	0.22 g
	Na_2SO_4	0.15 g
Solution 2:	$CaCl_2$	27.75 mg
(per 500 ml of distilled water)	$MgSO_4$	10.75 g
	NH_4VO_3	58.5 mg
	$ZnSO_4$	1.4375 g
Solution 3:	$CoCl_2.6H_2O$	23.8 mg
(per 500 ml of 0.05% HCl)	$CuSO_4.5H_2O$	1.25 mg
	$MnCl_2.4H_2O$	1 mg
	$NaMO_4.2H_2O$	60.5 mg
	$NiSO_4.6H_2O$	26.3 mg
Solution 4:		
(per 500 ml of 0.05% HCl)	$FeSO_4.7H_2O$	2 g
Solution 5:	Serine	10 g
(per 500 ml distilled water)	L-alanine	0.5 g
	L-arginine	0.5 g
	L-asparagine.H_2O	0.5 g
	L-aspartic acid	0.5 g
	L-glutamine	0.5 g
	L-glutamic acid	0.5 g
	Glycine	0.5 g
	L-histidine.HCl	0.5 g
	L-isoleucine	0.5 g
	L-leucine	0.5 g
	L-lysine.HCl	0.5 g
	L-methionine ‡	0.5 g
	L-phenylalanine	0.5 g
	L-proline	0.5 g
	L-threonine	0.5 g
	L-tryptophan	0.5 g
	L-valine	0.5 g
Solution 6:		
(per 100 ml distilled water)	Sodium pyruvate	10 g
Solution 7:		
(per 100 ml of distilled water)	α-ketoglutaric acid	10 g
Solution 8:	*i*-Inositol	100 mg
(per 500 ml of distilled water)	Thiamine.HCl	100 mg
	Calcium pantothenoate	100 mg
	Nicotinamide	50 mg
	Biotin	5 mg
Solution 9:		
(per 10 ml of 95% (v/v) ethanol)	DL-thioctic acid	10 mg
Solution 10:	Co-enzyme A	10 mg
(per 10 ml of distilled water)		
Solution 11:		
(per 5 ml of dilute NH_4OH)	Haemin †	10 mg

Construction of ABCD broth: (per 100 ml)		
	Solution 1	50 ml
	Solution 2	1 ml
	Solution 3	1 ml
	Solution 5	10 ml
	Solution 6	1 ml
	Solution 7	1 ml
	Solution 8	1 ml
	Solution 9	10 µl
	Solution 10	10 µl
	Cysteine.HCl ‡	50 mg
	Glutathione (reduced)	50 mg
	L-tyrosine	5 mg
	Solution 4	1 ml
	adjust pH to 6.5 using 20% (w/v) KOH	
	Solution 11	100 µl
	make up to 100 ml with distilled water	
	Filter sterilise with a sterile membrane filter (0.22 µm pore size; 25 mm diameter)	

Table 2.4: Composition and preparation of full ABCD broth and the constituent solutions. ‡ = methionine and cysteine are omitted from the broth used for labelling (ABCD broth minus methionine and cysteine).

Growth was enhanced when methionine was present in the ABCD broth, therefore as an additional supplement prior to incubation, 0.01% (v/v) Brucella broth was added to ABCD broth minus methionine and cysteine, providing the labelling medium.

2.5 Incubation and growth of *Campylobacter*

Plates were incubated at 37°C under a microaerobic atmosphere for 48h. A microaerobic atmosphere was created by replacing the atmosphere in a vacuumed anaerobic gas jar with an atmosphere consisting of 5% (v/v) O_2, 10% (v/v) CO_2, and 85% (v/v) N_2 from a commercial gas cylinder (BOC S957 220 L). Broth cultures were maintained at 37°C as 'starter' cultures, and subcultured every 48h. Starter cultures consisted of 10 ml Brucella-FBP broth in ½ oz bijoux bottles and were inoculated with a single isolated colony. The cap was tightly screwed and the culture incubated statically at 37°C for 12h (~10^9 cfu/ml).

For growth or stress experiments in Brucella-FBP broth, inoculation was with 10 µl of a 12h starter culture per 25 ml broth. Inoculation of the supplemented defined broths was with 25 µl (0.1%) of the starter culture per 25 ml. Cultures were incubated shaking at 120 revs min^{-1}, under a microaerobic atmosphere at 37°C for the designated period of time.

2.6 Growth of non-campylobacters

Other bacterial species (*E. coli*, *S. typhimurium*, *Klebsiella* sp, *S. aureus* and *B. subtilus*) were cultured in 10 ml Luria-Bertani broth (Table 2.5) and incubated overnight at 37°C. Cultures were maintained on Luria-Bertani agar plates incubated as for broth cultures.

Component	Quantity	
Bacto tryptone	10 g	
Bacto yeast extract	5 g	
NaCl	5 g	
Agar †	15 g	
Distilled water	to one litre	
		pH to 7.2 with NaOH
		Autoclave at 121°C for 20 min (15 psi)

Table 2.5: Composition of Luria-Bertani media (L-broth). † = absent from broth

2.7 Antibiotic sensitivity assays

Confluent growth was obtained by spread plating 200 µl of a 12h starter culture on to a dried Brucella-FBP agar plate. Sterile filter paper discs (5 mm diameter) soaked in the correct antibiotic concentration were then applied. The plates were incubated at 37°C for 48h under microaerobic atmosphere. A zone of inhibition greater than 10 mm in diameter indicated sensitivity.

Commonly used antibiotic stock solutions were prepared as described in Table 2.6.

Antibiotic	Solvent	Stock concentration
Chloramphenicol	Ethanol	34 mg/ml
Ampicillin	Water	100 mg/ml

Table 2.6: Commonly used antibiotic stock solutions

2.8 Preparation of buffers

Phosphate buffer (pH 5.8-8.0)

Mix 0.1 M $NaH_2PO_4.H_2O$ (13.8 g/l) and 0.1 M $Na_2HPO_4.7H_2O$ (26.8 g/l) in the ratio of 88:12, 39:61 or 5.3:94.7 to give a final pH of 6.0, 7.0 or 8.0 respectively. Adjust the volume to 200 ml with distilled water.

Tris-HCl buffer (pH 7.1-8.9)

To 25 ml 0.1 M Tris solution (12.1 g/l) was added the required volume of 0.1 M HCl to give the desired pH. The final volume was adjusted to 100 ml with distilled water.

Clark and Lubs buffer (pH 8.0-10.2)

To a 50 ml mixture of 0.1 M KCl (7.455 g/l) and 0.1 M H_3BO_3 (6.184 g/l), 20.8 or 43.7 ml of a 0.1 M NaOH solution was added to give a pH of 9.0 or 10.0 respectively. The volume was adjusted to 100 ml with distilled water.

Carbonate buffer (pH 9.2-10.8)

To make carbonate buffer at pH 9.0 and pH 10.0, 0.1 M $Na_2CO_3.10H_2O$ (28.62 g/l) and 0.1 M $NaHCO_3$ solutions (8.4 g/l) were prepared. The solutions were mixed in a ratio of 5:95 and 60:40 respectively in a total volume of 100 ml.

Phosphate buffered saline

PBS (pH 7.3) was prepared by the addition of one tablet of PBS (Oxoid Ltd.) to 100 ml distilled water. The resultant solution was autoclaved as described for the Brucella broth.

2.9 Sampling methods

2.9.1 Plate (viable) counts

Cells were serially diluted into Brucella broth, and 100 µl of the appropriate dilution plated out in duplicate. Plates were then incubated as previously described for 48h. The plate count was then calculated according to Equation 2.1:

$$cfu/ml = average\ number\ of\ colonies/\ (dilution\ factor \times volume\ plated\ in\ ml)$$

Equation 2.1: Calculation of the plate count expressed in colony forming units per ml

2.9.2 Epifluorescent microscopy and photography of cells

The microscope was a Leitz-Wetzlar Mettallux II fluorescent microscope with a mercury lamp, fitted with blue (setting 1), green (setting 3) and red (setting 4) filters for excitation of fluorescent dyes, as well as bright-field (setting 2). Slides were generally examined under oil immersion at ×100 phase contrast under setting 2. Various fluorescent dyes could be observed via the different filters: DAPI (blue), CFW (blue), CFDA (green), Rh123 (green), PI (red). Photographs were taken using the same microscope with ISO400 black and white film (Ilford 400 Delta Professional; 36 exposures). Negatives were developed by the University of Edinburgh photography department.

2.9.3 Total count: DAPI/ membrane filter method

Millipore polycarbonate filters (0.22 μm pore size) were pre-stained (Hobbie, *et al.*, 1977) for 24h in a 0.2% (w/v) Irgalan black solution (0.2 g Irgalan black per 100 ml 2% (v/v) acetic acid/ 2.5% (w/v) glutaraldehyde solution). The stained filters were then washed in sterile water and arranged in a Swinnex filter holder, and autoclaved as for the media.

A 1 ml volume of cells was pelleted at 13,000 *g* for 5 min in a bench centrifuge. The supernatant was discarded and the pellet resuspended in 1 ml PBS (pH 7.3). Then, 5 μl of DAPI (0.1 mg/ml stock) was added, giving a final concentration of 1.5 μM, and incubated for 30 min at room temperature. Depending on the initial density, the cell suspension was diluted to give a total volume of 1 ml in PBS (pH 7.3) according to Table 2.7:

Approximate concentration	Dilution factor
10^{10} cells/ml	1000
10^{9} cells/ml	100
10^{8} cells/ml	10
10^{7} cells/ml	None

Table 2.7: Dilution of cells for the DAPI total count method

The diluted, stained cells were added to 4 ml PBS (pH 7.3). The suspended cells (5 ml) were filtered slowly through a black pre-stained Millipore membrane filter (0.22 μm pore size) to achieve even distribution of the cells on the surface of the filter. The filter was removed and dried before being placed onto a slide with the cell-side facing upwards. A drop of Mowiol (50 μl; Calbiochem) was added to the centre of the filter, and a coverslip pressed firmly on top until the Mowiol solidified. Slides could be stored for up to one week at 4°C without loss of fluorescence. The slide was

examined as indicated in Section 2.9.2. The numbers of cells in 20 fields of view were counted, and the total count calculated from Equation 2:

cells/ml = average number of cells per field $\times$ *22500*† $\times$ *(1000/ volume of cells filtered in μl)*

Equation 2.2: Calculation of the total count via the membrane filter method. † = the number of fields of view per membrane was calculated from the usable area of the membrane divided by the area of a field of view. The usable area of a 25 mm membrane filter is 346 mm^2. The area of a field of view at ×100 magnification was 15.4 μm^2.

2.9.4 Morphological counts

Cells could be preserved for up to 2 months by the addition of 0.2% (v/v) formalin and storage at 4°C. Initially, 450 μl of Brucella agar was dispensed and spread evenly at the centre of a clean microscope slide. Once set, 50 μl of diluted cells (2:1 ratio of cells to broth) were added to the centre of the agar and allowed to adsorb to the surface at room temperature. A clean coverslip was then overlaid, before the cells were examined as described in Section 2.9.2. Morphological forms were recorded from twenty fields of view (~500 cells) and the percentages calculated.

2.9.5 Chromosome staining

Method 1:

A slide was prepared with agar as for the morphological count (Section 2.9.4). Cells (1 ml) were pelleted and resuspended in 1 ml PBS (pH 7.3) before addition of 200 μg/ml chloramphenicol for 10 min to allow chromosome condensation. Cells were stained for 30 min with 1.5 μM DAPI. The diluted cells were added to the agar on the slide and allowed to adsorb to the surface at room temperature. A clean coverslip was then overlaid, before the cells were examined as described in Section 2.9.2.

Method 2 (Hiraga *et al.*, 1989):

A 1 ml volume of cells was pelleted and washed twice with PBS (pH 7.3)., before the addition of chloramphenicol to a final concentration of 200 μg/ml for 10 min. A clean slide was wiped with HCl, and 15 μl of the washed cells dispensed in the centre. The slide was dried at room temperature. The dried cells were washed in methanol, and left to dry at room temperature for 5 min. The slide was washed six times by immersing in a beaker of tap water, and dried on paper towels, cell side up. To the cells, 10 μl poly-L-lysine (5 μg/ml stock in distilled water) was added and spread over the surface of the cells with a sterile pipette tip. The cells were dried at room temperature, before 10 μl of DAPI was added (5 μg/ml stock). A clean coverslip was overlaid, and the cells visualised as described in Section 2.9.2.

2.9.6 Gram's Stain

A thin smear of bacterial suspension was dried onto a clean slide, and fixed by passing through a flame three times. The slide was stained with crystal violet solution for 5 min, excess stain decanted and then incubated with Lugol's iodine solution for 2 min. The iodine solution was drained off the slide and washed with isopropanol-acetone (3:1 v/v) or 95% (v/v) ethanol, followed by a wash with distilled water. The slide was counter-stained with 0.5% (w/v) basic fuchsin or safranin for 30 s, washed in water, and blotted dry. Cells were observed under phase contrast as described in Section 2.9.2.

2.9.7 Calcofluor white (CFW) assay

To 100 μl of cells, 5 μl of a 0.1 mg/ml stock solution of CFW was added, giving a final concentration of 5 μM. The sample was then incubated for 5 min at

37°C. The cells were fixed onto a slide (Section 2.9.4) and visualised (Section 2.9.2) using the blue filter.

2.9.8 INT assay

To 100 µl of cells, 5 µl of a 10 mM stock solution of INT was added, giving a final concentration of 0.5 mM. To stimulate respiration, 1 µl of a 20% (w/v) succinate solution was added, giving a final concentration of 10 mM succinate. Two controls were used: one omitting succinate, and one with boiled cells. The samples were incubated for 10 min at 37°C. The cells were fixed onto a slide (Section 2.9.4) and visualised under the microscope (Section 2.9.2) using the bright field setting. INT reduction could be observed as accumulation of optically dense, red inclusions within the cells.

2.9.9 Rhodamine 123 (Rh123) assay

Cells (500 µl) were pelleted at 13,000 *g* for 5 min in a bench centrifuge. The supernatant was decanted and the cell pellet was resuspended in 500 µl PBS (pH 7.3). Then EDTA (5 µl 10 mM) was added and the 500 µl was aliquoted into 100 µl volumes. Rh123 (5 µl 50 µg/ml) was added to a 100 µl volume, giving a final concentration of 2.5 µg/ml. Cells were incubated for 30 min at 37°C. The cells were fixed onto a slide (Section 2.9.4) and visualised by fluorescent microscopy (Section 2.9.2) using the green filter.

2.9.10 Carboxyfluorescein diacetate (CFDA) assay

Cell cultures were divided into two 1 ml aliquots, and spun at 13,000 *g* for 5 min in a bench centrifuge. The pellet was resuspended in 1 ml 100 mM phosphate/ 1 mM EDTA buffer (pH 6.7). To each aliquot, 2 µl of a 0.5 mg/ml CFDA solution was

added, giving a final concentration of 1 μM, and the suspension mixed by inversion, and then incubated for 90 min at 37°C. Cells were spun in a bench centrifuge for 5 min. The supernatant (850 μl per aliquot) was removed and dispensed into a fluorometric glass cuvette. A blank containing 4 μl CFDA in a total of 1.7 ml 100 mM phosphate/ 1mM EDTA buffer (pH 6.7) was always included, and the value deducted from the sample reading. Fluorescent intensity was measured on a spectrofluorimeter (Perkin-Elmer) set at an excitation wavelength of 492 nm with a 5mm slit, and an emission wavelength range of 490 to 560 nm with a 2.5 mm slit. Readings were always recorded at 513 nm, the maximum emission wavelength for CFDA.

2.9.11 Propidium iodide (PI) assay

To 100 μl of cell suspension, 5 μl of propidium iodide (0.1 mg/ml stock) was added giving a final concentration of 5 μg/ml. Cells were incubated at 37°C for 10 min. Stained cells were viewed as described in Section 2.9.2 under the red filter, using slides prepared as described in Section 2.9.4.

2.9.12 Activity dye stock solutions

The various dyes were prepared as stock solutions according to Table 2.8:

Chemical	Solvent	Stock concentration
Calcofluor white	Sterile water	0.1 mg/ml
INT	Sterile water	10 mM
Rhodamine 123	Sterile water	50 μg/ml
CFDA	Acetone	0.5 mg/ml
Propidium iodide	Sterile water	0.1 mg/ml
DAPI	Sterile water	0.1 mg/ml
Acridine orange	Sterile water	0.1 mg/ml

Table 2.8: Stock solutions of dye solutions

Other solutions used in the dye assays were prepared as follows:

10 mM EDTA stock: 29.2 mg EDTA, disodium salt
10 ml distilled water

20% (w/v) succinate: 2 g sodium succinate
10 ml distilled water

100 mM phosphate/ 1 mM EDTA buffer (pH 6.7):

499 ml 100 mM phosphate buffer (pH 6)
1 ml 0.5 M EDTA solution

2.9.13 Sphaeroplast preparation by the lysozyme-EDTA method (modification of Birdsell & Cota-Robles, 1967)

Pelleted cells (1 ml) were resuspended in 10 mM Tris-HCl/ 0.5 M sucrose (pH 8.0), to a final concentration of 10^8 to 10^9 cells/ml, divided into two 500 µl aliquots and incubated at room temperature for 10 min. To each aliquot, lysozyme (30 mg/ml stock) was added to a final concentration of 200 µg/ml (*C. jejuni*; 3.35 µl per 500 µl) or 30 µg/ml (*E. coli*; 0.5 µl per 500 µl) and incubated at room temperature for a further 10 min. An equal volume (500 µl) of 10 mM Tris-HCl (pH 8.0) was added and incubation continued for a further 10 min before the addition of EDTA (4 µl 0.5 M stock) giving a final concentration of 2 mM. Sphaeroplast formation was monitored via microscopy throughout. Once >90% of the cells were spherical or if lysis was evident as an increase in viscosity, the reaction was terminated by adding $MgSO_4$ (10 µl 0.5 M stock) to give a final concentration of 5 mM.

The solutions used in the protocol were constructed as follows:

10 mM Tris-HCl (pH 8.0):	0.12 g Tris 50 ml distilled water

The pH was adjusted with HCl to 8.0 and the solution made up to a final volume of 100 ml with distilled water.

10 mM Tris-HCl/ 0.5 M sucrose (pH 8.0):	100 ml 10 mM Tris-HCl (pH 8.0) 17.12 g sucrose
0.5 M EDTA:	1.46 g EDTA 10 ml distilled water
0.5 M $MgSO_4$:	0.6 g $MgSO_4$ 10 ml distilled water

2.9.14 Flow cytometry

The instrument used was a Beckman-Coulter XL flow cytometer, processing at a flow rate of 10,000 cells/s. Cells were prepared in phosphate buffered saline containing 2% (v/v) formaldehyde to a concentration of 10^7 cells/ml. A total of 500,000 cells were counted for each sample processed. Data was collected on a two-parameter histogram displaying forward scatter (cell size) against side scatter (cytoplasmic complexity).

2.10 Protein protocols

2.10.1 Protein quantification assay (modified Lowry assay, Peterson, 1977)

Preparation and precipitation of protein samples

Protein samples were diluted in sterile water to give a concentration of approximately 50-200 μg/ml in a final volume of 200 μl (usually at 10 and 100-fold initial dilution). Sodium deoxycholate (50 μl of 0.15% (w/v) solution) was added to each sample. Samples were vortexed and incubated at room temperature for 10 min before addition of 50 μl 72% (w/v) TCA solution. After mixing, samples were immediately centrifuged for 15 min at 13,000 *g*. The supernatant was removed and the pellet resuspended into 200 μl of sterile water. This method involves precipitation of proteins to reduce the effects of interfering substances.

Preparation of standards

Standards, giving final concentrations of 50, 100, 150 and 200 μg/ml BSA, were prepared in a final volume of 200 μl. These were used to establish a standard curve.

Colour production

Freshly prepared reagent A (600 μl) was added to each of the samples and standards. The tubes were vortexed briefly and incubated for 30 min at room temperature. To each tube was added 100 μl Folin & Coicalteu's phenol reagent (reagent B), and the solutions were incubated at room temperature for 45 min after mixing.

Measurement of standard curve and calculation of sample concentration

The absorbance at 660 nm (A_{660}) was measured, and the protein concentrations were determined from the BSA standard curve plot of A_{660} versus μg/ml standard protein.

The solutions used during the protocol were constructed as indicated below:

0.15% (w/v) sodium deoxycholate:
0.15 g sodium deoxycholate
100 ml distilled water

72% (w/v) Tricarboxylic acid:
7.2 g TCA
10 ml distilled water

1.6% (w/v) Sodium tartrate:
1.6 g sodium tartrate
100 ml distilled water

4% (w/v) Sodium hydroxide:
4 g NaOH
100 ml distilled water

4% (w/v) Copper sulphate:
0.4 g $CuSO_4.7H_2O$
10 ml distilled water

Reagent A:
2 ml 10% (w/v) SDS stock
2 ml 1.6% (w/v) sodium tartrate
2 ml 4% (w/v) NaOH
0.2 ml 4% (w/v) $CuSO_4.7H_2O$
13.8 ml distilled water
0.4 g sodium carbonate

Reagent B:
2.5 ml Folin's & Coicalteu's reagent
2.5 ml distilled water

2.10.2 One-dimensional (1D) SDS-polyacrylamide gel electrophoresis

All 1D-PAGE analysis was performed using a Mini-Protean II dual slab cell (Bio-Rad) capable of running two mini-gels with 0.75 mm spacers, 10 well combs (maximum well volume = 27 µl) and glass plates (dimensions: inner = 730 × 102 mm; outer = 830 × 102 mm). All solutions were made according to the Laemmli buffer system (Laemmli, 1970). The compositions of the solutions are shown at the end of the section.

Assembly of gel

The gel plates were assembled according to the manufacturers instructions, and filled within 1 cm of the top, initially with 12% resolving gel, and overlaid with distilled water. Once set (~60 min) the water overlay was removed, and the dried resolving gel washed three times with distilled water to remove any unset acrylamide. The comb was placed, leaving a 2-3 mm gap between the bottom of the comb, and the top of the resolving gel. A 4% stacking gel was then overlaid on top of the resolving gel to create the wells. Once set, the comb was removed, and the wells washed with distilled water. The complete gel was then constructed in the gasket/ cooling core apparatus according the manufacturers instructions. The upper chamber and lower chambers were filled with running buffer.

Preparation of protein samples and electrophoresis

Samples were diluted 1:4 with SDS-lysis buffer. Markers were reconstituted in SDS-lysis buffer according to the listed solutions. Both samples and markers were boiled at 100°C for 5 min. The loading volumes were 25 µl for protein sample and 10 µl for the molecular weight markers. The gels were run at 150 V for 45 min at room

temperature. Visualisation of proteins was by either Coomassie stain (Section 2.10.4.1), silver stain (Section 2.10.4.2) or autoradiography (Section 2.10.7).

Solutions used in performing 1D-SDS-PAGE

Resolving gel (12% acrylamide):	(per 10 ml)
Distilled water	3.35 ml
1.5M Tris HCl (pH 8.8)	2.5 ml
10% (w/v) SDS stock	100 µl
30% (w/v) acrylamide/bisacrylamide stock	4.0 ml
	Added at time of use:
10% (w/v) APS	50 µl
TEMED	5 µl

Stacking gel (4.0%):	(per 5 ml)
Distilled water	3.05 ml
0.5 M Tris-HCl (pH6.8)	1.25 ml
10% (w/v) SDS stock	100 µl
30% (w/v) acrylamide/bisacrylamide stock	0.65 ml
	Added at time of use:
10% (w/v) APS	50 µl
TEMED	10 µl

SDS-lysis sample buffer:	(per 8 ml)
Distilled water	4.0 ml
0.5 M Tris-HCl (pH 6.8)	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS stock	1.6 ml
0.05 % (w/v) bromophenol blue	0.2 ml
2-β-mercaptoethanol (added prior to use)	0.4 ml

5X Electrode (running) buffer (pH 8.3):

Tris base	9 g
Glycine	43.2 g
SDS	3 g

made up to 600 ml with distilled water and stored at 4°C. Before the stock solution was diluted by adding 60 ml 5X running buffer to 240 ml distilled water.

1D-PAGE Markers:

SDS-lysis buffer	170 µl
Bovine serum albumin	10 µl
Carbonic anhydrase	10 µl
Ovalbumin	10 µl

2.10.3 Two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975)

The first dimension equipment consisted of the Multiphor II Electrophoresis unit, the Immobiline® DryStrip kit (anodic and cathodic electrodes, gel tray, strip aligner, and IEF electrode strips; Pharmacia), and the Reswelling tray (Pharmacia). The second dimension was performed using the Hoefer™ SE 600 (standard vertical) system for 14 × 15 cm gels. The gels were then scanned, and the resultant images analysed by 2D Gel Analysis Software (Phoretix; Non-Linear Dynamics Ltd).

Growth of cells for metabolic labelling

A 25 ml volume of ABCD broth (minus met and cys) was supplemented with 0.01% (v/v) Brucella broth, and inoculated with 100 µl of a 12h starter culture. Cultures were incubated for approximately 16h until the cells were in logarithmic phase of growth (10^8 to 10^9 cells/ml). Microscopy was used to measure the total cell count and to check culture purity and cell morphology.

Metabolic labelling

Cells were dispensed into 1 ml volumes (three per time-point), and incubated in pre-cooled or warmed gas jars containing 500 ml of water and a float, under microaerobic atmosphere. The cells were incubated for the required period of time under the specific experimental parameters (usually cold-shock at either 4, 25 or 32°C; 0.1 mM H_2O_2 or 32 mM TSP).

After incubation, the three 1 ml volumes for the specific time-point were removed, and the cells were metabolically labelled with 15 μCi/ml [^{35}S]-methionine. The cell suspension was vortexed briefly, and incubated for 30 min at the relevant temperature to avoid oxidative shock. After centrifugation for 1 min at 13,000 *g* the supernatant was removed, and the pellets stored at -80°C until use for sample preparation. One dimensional-PAGE was performed on one sample to confirm metabolic labelling of the proteins. The radiolabelled proteins were visualised using a phosphoimaging system (Molecular Dynamics).

Sample preparation

The pellets were resuspended in 600 μl of sonication buffer, before sonication using a slim probe (Lucas Dawe Ultrasonics; Soniprobe*)*, consisting of three 20 s pulses at 10 s intervals to avoid excessive heating. Samples were removed (50 μl) for protein concentration determination (Section 2.10.1), before addition of 25 μl 1mg/ml DNase and incubation on ice for 5 min. Protein solubilisation was aided by increasing the urea concentration to 9M by the addition of 0.3g urea. Then 2% (v/v) Nonidet P40 was added to aid solubilisation of membrane proteins, and incubated at room temperature for 5 min with gentle inversion. The sample were stored at -20°C until further processing.

Rehydration of IEF strips

The samples were diluted accordingly so that 10 μg protein was loaded per strip. IEF strips (Immobiline™ Drystrip) were rehydrated in the Immobiline™ Drystrip Reswelling Tray (Pharmacia). The Reswelling Tray was levelled and the diluted sample and rehydration solution mixed in a 1:1 ratio to give a final volume of 240 μl. The sample/rehydration solution was added to the designated reservoir slot, and the IEF strips placed gel-side down in the reservoir slot. The IEF strips were overlaid with Plusone™ DryStrip Cover Fluid to prevent sample evaporation and urea crystallisation. The protective lid was replaced onto the Reswelling Tray, and the samples rehydrated for at least 12h at room temperature.

First dimension: Electrophoresis of IEF strips

The cooling unit was switched on to heat the Multiphor II unit to 15°C. Cover Fluid (5 ml) was smeared over the surface of the heating plate, and the Gel Tray laid on top with the removal of any air bubbles, therefore creating good contact between the two surfaces. The anodic and cathodic electrodes were connected and mineral oil (5 ml) was smeared on top of the Gel Tray. The Strip Aligner was then placed on top (12 grooves side up) of the Gel Tray with care to avoid getting Cover Fluid on the Strip Aligner surface. Two 11 cm pieces of IEF Electrode Strips were soaked with distilled water, and blotted thoroughly to prevent streaking.

The rehydrated IEF strips were removed from the Reswelling Tray, and the backs were blotted to remove excess Cover Fluid. The IEF strips were placed gel-side up, in the centre of the Strip Aligner grooves, and aligned so that all of the basic ends were level, and the acidic ends (pointed) facing towards the anode. The acidic and

basic ends of the IEF strips were partially covered with the blotted IEF Electrode Strips, before the correct electrode was placed on top of the IEF Electrode strip, with gentle pressure to ensure adequate contact. The Gel Tray was filled with Cover Fluid before the Mulitphor II unit lid was replaced and the electrodes connected to the power unit. The IEF strips were initially run at 0.3 kV at 18 mA and 15 W for 2h (0.6 kV/h total), and then 1.4 kV at 30 mA and 35 W for 22h (30,000 kV/h total) overnight.

First dimension: Freezing IEF strips and cleaning the first dimension equipment

The power pack was switched off and the electrodes removed, the oil drained, and the wicks discarded. The strips were removed from the Multiphor II unit, and gently blotted to remove excess oil. The IEF strips were then placed on pieces of labelled parafilm, and wrapped in Saran Wrap before storage at -20°C, until the second dimension is run. The remaining mineral oil was poured from the Gel Tray into a glass beaker, and the Gel Tray, electrodes, and Strip Aligner cleaned in a radioactive room, using hot water, and Decon. The heating plate was then wiped and the remaining Cover Fluid was filtered back into a container for re-use.

Second dimension: Preparation of polyacrylamide gels

The gel plates and spacers were constructed according to the manufacturers instructions. Usually gel sandwiches were constructed permitting the simultaneous electrophoresis of four slab gels, and in all cases 1 mm spacers were used.

The resolving gels (12%) were poured one at a time to a depth of 13.5 cm and overlaid with water-saturated butanol, before leaving to set for 45 min. The water-saturated butanol was removed, and the gels washed with distilled water to remove unpolymerised acrylamide. Any excess water was then removed by blotting with filter

paper. The stacking gels (4%) were poured to a depth of 1 cm, producing a final gel depth of 14.5 cm, before being overlaid with water-saturated butanol, and left to set for 45 min.

Second dimension: Equilibration of the IEF strips

Equilibration solution A (15 ml per IEF strip) and equilibration solution B (15 ml per IEF strip) was dispensed into boiling tubes. The IEF strips were defrosted for 5 min prior to use. The strips were placed into equilibration solution A with gentle shaking for 10 min, before removal and placement into equilibration solution B for 10 min with shaking. The strips were then removed and gently blotted on filter paper to remove excess equilibration solutions.

Second dimension: Addition of equilibrated IEF strips to the slab gels

The water-saturated butanol was poured from the set stacking gel, and the gel rinsed with distilled water. The tops of the gels were then dried by gently blotting with filter paper. The equilibrated IEF strips were placed on top of the stacking gel, leaving space at the acidic end to place the markers (on filter paper). The IEF strip and markers were sealed with a 1% (w/v) agarose solution.

Electrophoresis of the second dimension

The upper gel tank was placed on top of the gels according to the manufacturers instructions. The gels were then placed inside the gel tank and 4.5 L of running buffer was added to the lower chamber. The upper chamber was filled with 0.5 L running buffer. The lower chamber was then filled to its maximum level, before the electrodes were connected. The 1 mm thick gels were run at constant ampage of 12 mA per gel for 16h or until the dye front had reached the bottom of the gel.

Solutions used during 2D-PAGE

Sonication buffer (pH 7.4):

80 ml 10 mM Tris-HCl (pH 7.4)
0.102 g $MgCl_2.6H_2O$
500 µl pancreatic RNase (10 mg/ml)
to 100 ml with 10 mM Tris-HCl (pH 7.4)

Rehydration solution:

2.7 g urea
0.1 g NP-40
100 µl IPG buffer (pH 3-10L)
15 mg Dithiothreitol
1 grain Bromophenol blue
to 5 ml with distilled water

12% resolving gel solution:
(per 9 gels)

53 ml distilled water
39.4 ml 1.5 M Tris-HCl (pH 8.8)
1.575 ml 10% (w/v) SDS
63 ml bis/acrylamide

at the time of pouring add (per 25 ml):

145 µl 10% (w/v) APS
15 µl TEMED

4% stacking gel solution:
(per 9 gels)

28 ml distilled water
5 ml 0.5 M Tris-HCl (pH 6.8)
0.4 ml 10% (w/v) SDS
6.4 ml bis/acrylamide

at the time of pouring add (per 10 ml):

30 µl 10% (w/v) APS
10 µl TEMED

Equilibration solution (basis):	20 ml 0.5 M Tris-HCl (pH 6.8)
(per 200 ml)	72 g urea
	60 ml glycerol
	2 g SDS
	67 ml distilled water
Equilibration solution A:	60 ml equilibration solution
(per 60 ml; 4 IEF strips)	0.151 g dithiothreitol
Equilibration solution B:	60 ml equilibration solution
(per 60 ml; 4 IEF strips)	2.71 g iodoacetamide
	1 grain bromophenol blue
1% (w/v) agarose solution:	0.2 g agarose
(per 20 gels)	20 ml distilled water
Running buffer:	144 g glycine
	30 g Tris base
	5 g SDS
	make up to 5 L with distilled water
2D-PAGE markers:	17.5 µl Bio-Rad markers
	50 µl β-mercaptoethanol
	932.5 µl 5 × running buffer

Boil the markers for 5 min at 100°C. Add two grains of bromophenol blue. Store at 4°C until further use. Dispense 20 µl onto filter paper (0.5 cm^2).

2.10.4 Protein stains

Generally for 1D-gels the Coomassie blue stain was used unless otherwise stated. Silver staining (Blum *et al.*, 1987) was used for 2D-PAGE unless the gels were electroblotted or used for tryptic digestion, when GelCode Blue was used (Section 2.16.3). For 1D- and 2D-gels, 100 ml and 200 ml of the various staining solutions were used respectively.

2.10.4.1 Coomassie Blue

The gels were stained for 30 min in fixative, with gentle shaking, before the fixative was poured off and kept for future use (up to 5 times). The gels were destained for 1-3h with gentle shaking. Optionally, a foam bung was added to facilitate the removal of any Coomassie stain that leaches out of the gel.

Fixative (per 100 ml):

50 ml distilled water
40 ml methanol
10 ml glacial acetic acid
0.1 g Coomassie Blue R-250

Destain (per 100 ml):

50 ml distilled water
40 ml methanol
10 ml glacial acetic acid

Sequential destaining was sometimes performed to permit additional silver staining by the following protocol:

Solution	Incubation Time
40% (v/v) ethanol; 5% (v/v) acetic acid	1-2h
30% (v/v) ethanol; 5% (v/v) acetic acid	1-2h
30% (v/v) ethanol; 5% (v/v) acetic acid	1h
20% (v/v) ethanol; 5% (v/v) acetic acid	1h
10% (v/v) ethanol; 5% (v/v) acetic acid	16h

2.10.4.2 Silver Stain (Blum *et al.*, 1987)

The proteins were fixed by washing the gel in fixative three times each for 30 min. The gels were then rinsed in 20% (v/v) ethanol for 10 min, followed by a 10 min rinse in distilled water. The gel was then washed for 60 s with the sensitising solution, prior to rinsing three times for 20 min, with distilled water. The gel was then impregnated with silver, by incubation for 45 min with the staining solution. The gel was then rinsed for 10 min in distilled water and developed for 30 min in the developing solution or until the protein bands/spots appeared. Developing was terminated by a 30 min incubation in the stop solution. The gel was gently shaken throughout the staining process.

Fixative (per litre):	300 ml ethanol 100 ml glacial acetic acid
20% (v/v) ethanol (per litre):	200 ml ethanol
Sensitiser solution (per litre):	0.2 g sodium thiosulphate
Staining solution (per litre):	2 g silver nitrate 0.7 ml 35% (v/v) formaldehyde

Developer (per litre):

30 g sodium carbonate
10 mg sodium thiosulphate
0.25 ml 35% (v/v) formaldehyde

Stop solution (per litre):

50 g Tris base
25 ml glacial acetic acid

2.10.4.3 Drying gels

The gels were rinsed in water several times before placement for 3h into a solution consisting of 35% (v/v) ethanol and 10% (v/v) glycerol. The gels were then placed on filter paper (Whatman), and overlaid with Saran wrap, before being dried on a Slab Gel Dryer SGD2000 (Savant) for 90 min (mini-gels) or 8h (full gels; 1mm thick).

Drying solution (per litre):

350 ml ethanol
100 ml glycerol

2.10.5 Electroblotting

Electroblotting of 2D-PAGE was performed using Trans-Blot® Electrophoretic Transfer Cells, blotting cassettes, and fibre pads (Bio-Rad). Each cell required approximately 2.5 L of transfer buffer and was capable of electroblotting two gels. Due to the negative charge of the SDS bound proteins, application of an electric field permits migration from the cathode to the anode, and subsequent trapping in the nitrocellulose membrane.

Equilibration of gels

The electroblotting cell was prepared by rinsing with distilled water, before the cassettes were placed into the cell. The cell was then filled with 1× transfer buffer. The transfer buffer was prepared just before use to prevent methanol evaporation.

The gel was prepared by disassembling the gel apparatus, removing the stacking gel, and cutting a small piece off of the corner above the markers for orientation. The gels were equilibrated in 200 ml 1× transfer buffer for the designated length of time, dependent on gel thickness (0.5 mm = 1 min; 0.75 to 1 mm = 5 min; 1.5 mm = 15 min). This removed excess SDS, permitting transfer of proteins within an electric field. Longer equilibration removed too much SDS, decreasing transfer efficiency. Cut several pieces of filter paper (3 per gel) and nitrocellulose membrane (1 per gel), to 0.5 mm greater than the dimensions of the gel. Soak in transfer buffer just prior to use.

Transfer of gel to nitrocellulose membrane sandwich

A wetted fibre pad was placed on the black (cathode) surface of the blotting cassette. Then two pre-wetted pieces of filter paper were placed on top, followed by the equilibrated gel. The pre-wetted nitrocellulose membrane was then placed on top of the gel, followed by an additional piece of pre-wetted filter paper. After each addition to the sandwich any air bubbles were gently removed. The second fibre pad was then added, and the completed blotting cassette closed. The nitrocellulose membrane should be facing the anodic side of the cassette to ensure the correct orientation of the electric field.

Conduct of electro-transfer

The assembled blotting cassette was inserted into the electroblotting cell, with the nitrocellulose membrane facing the anodic side. The cell was filled so that the transfer buffer completely covered the electrode panels (but does not touch the electrical connections). The apparatus was then transferred to 4°C before the cooling tube was connected to cold running water to prevent overheating. The power supply was connected and the transfer was performed at a constant current (200 mA) for the specified time, depending on gel thickness (0.5 mm = 1h; 1 mm = 2h; 1.5 mm = 3h).

At the end of transfer, the power supply was disconnected, and the sandwich was opened and the membrane and gel removed. The membrane was washed for 5 min (three times) with distilled water, before being dried at room temperature and subjected to the Enhanced Autoradiography system (Section 2.10.6). The efficiency of protein transfer can be confirmed by staining of the electroblotted gel (Section 2.10.4.1). Solutions used in the electroblotting protocol were prepared as follows:

×**20 stock Tris/ glycine buffer** (one litre):	150.14 g glycine 24.228 g Tris
×**1 transfer buffer** (four litres):	200 ml ×20 stock buffer 400 ml methanol

2.10.6 Enhanced Autoradiography system

Introduction

Conventional autoradiography involves detecting emitted β-particles during the decay of radioisotopes on X-ray film, whence they emit light. Only those emissions with a trajectory towards the film are detected. Enhanced Autoradiography is approximately 50-fold more sensitive, partly due to the creation of a transparent

sample membrane via EA-wax application, allowing conversion of a higher percentage of emissions into detected light.

Protocol

After protein transfer onto nitrocellulose membranes, the membranes were dried at room temperature. A dried membrane was placed on top of a sheet of silicone release material (SRM) possessing no wax, and pieces of EA-wax were placed upon the membrane, at approximately 4 cm intervals. Another sheet of SRM was then placed on top, creating a SRM:membrane:EA-wax:SRM sandwich. The sandwich was then placed between two pre-heated glass plates, and clamped along each edge with bulldog clips. The assembly was then placed at 65°C for 5 min to allow the EA-wax to permeate the entire membrane. The assembly was removed from the oven, and placed at 4°C for 3 min, before removal of the bulldog clips and glass plates, enabling the separation of the sandwich. The membrane should be evenly impregnated with EA-wax. If areas are devoid of wax, repeat the procedure, placing wax on the unimpregnated parts. Successful impregnation was observed when text could be read when placed beneath the membrane.

An autoradiography cassette was covered with aluminium foil (shiny side upwards), and the labelled impregnated membranes placed on top and fixed into place with Glogos™ II Autorad Markers (Stratagene). The membranes were then clearly labelled. In a darkroom a sheet of 'pre-flashed' X-ray film (Agfa-Gavaert N.V.) was placed over the impregnated membranes and the cassette was closed. The reflective side of the foil should be facing the film, thereby increasing detection efficiency. The cassette was then placed at -70°C for 4 days and developed according to Section 2.10.7. EA-wax can be removed from membranes by washing in toluene, xylene or their substitutes, permitting staining of protein spots.

2.10.7 Autoradiography

The sample was fixed into an autoradiography cassette using Glogos™ Autorad Markers. The markers were charged by shining light on them prior to use. In the dark, a sheet of 'pre-flashed' X-ray film (Afga-Gavaert N.V) was placed over the sample, and the cassette closed. The cassette was stored for a period of time at -70°C, depending on the strength of labelling. For [^{35}S]-labelling with Enhanced Autoradiography, the incubation period was 4 days, whilst without the treatment it was 4 weeks. After the required incubation period the autoradiographs were developed on an X-ograph Compact X2 autoradiograph developing machine

2.11 Quantitation of hypochlorite and hydrogen peroxide concentrations

2.11.1 Hypochlorite ions

Hypochlorite ions oxidise colourless iodide ions to iodine (red-brown), with the amount of iodine produced proportional to the hypochlorite concentration according to Equation 2.3. A 20% (v/v) bleach solution was prepared in a volumetric flask. In a beaker, precisely 10 ml of the 20% (v/v) bleach solution was acidified with 10 ml 1M H_2SO_4, and reacted with 10 ml 0.1M KI solution.

$$ClO^-_{(aq)} + 2I^-_{(aq)} + 2H^+_{(aq)} \longrightarrow I_{2\,(aq)} + H_2O_{(aq)} + Cl^-_{(aq)}$$

Equation 2.3: Chemical equation depicting the oxidation of iodide ions by hypochlorite ions

The resultant iodine solution was titrated against 0.1M sodium thiosulphate. Thiosulphate ions reduce iodine to colourless iodide ions, as shown in Equation 2.4, indicating the end-point. The sensitivity of the reaction could be increased by adding a few mls of a 1% (w/v) starch solution before the end-point is reached.

$$2S_2O_3^{2-}{}_{(aq)} + I_{2\,(aq)} \longrightarrow 2I^-_{(aq)} + S_4O_6^{2-}{}_{(aq)}$$

Equation 2.4: Chemical equation depicting the reduction of iodine by thiosulphate ions

2.11.2 Hydrogen peroxide

Hydrogen peroxide oxidises the purple manganate (VII) ions to colourless Mn^{2+} ions (end-point), according to Equation 2.5. A 5% (v/v) hydrogen peroxide solution was prepared in a volumetric flask. In a beaker, precisely 10 ml of the diluted hydrogen peroxide solution was acidified with 10 ml 1M H_2SO_4, and titrated against a 0.1M $KMnO_4$ solution.

$$5H_2O_{2\,(aq)} + 2MnO_4^-{}_{(aq)} + 6H^+{}_{(aq)} \longrightarrow 5O_{2\,(g)} + 2Mn^{2+}{}_{(aq)} + 8H_2O_{(aq)}$$

Equation 2.5: Chemical equation depicting the oxidation of manganate (VII) ions by hydrogen peroxide

2.11.3 Calculation of the unknown concentration

For both titrations, the volume of the titrate was determined from the experiment. Therefore, using the formula below, the number of moles of thiosulphate or manganate (VII) ions can be determined:

$$moles = (volume/1000) \times concentration$$

Equation 2.6: Formula comparing moles, volume and concentration of a solution. 1M = 1 mol dm^{-3}.

The numbers of moles of hypochlorite ions or hydrogen peroxide in the diluted solution were determined from Equations 2.3 and 2.5. Therefore, once the numbers of moles were known, the concentration could be calculated. The percentage concentration of the hypochlorite or hydrogen peroxide solutions was determined according to equation 2.7.

$$\%\ concentration = (concentration \times M_r)/\ 10$$

Equation 2.7: Calculation of the percentage component in the undiluted solution. Note: concentration (M) × M_r is equal to concentration in grams per litre.

2.12 Stress application: oxidative stress and cold-shock

Cultures were grown as previously described (Section 2.5), under optimal growth conditions for the designated period of time, before subjection to the appropriate stress condition:

(a) **Oxidative shock (aeration)**: cultures were shaken (120 revs min^{-1}) under aerobic atmosphere at the appropriate temperature.

(b) **Cold-shock**: cultures were incubated statically at the stress temperature (4, 20, 25, or 32°C). If a microaerobic atmosphere was required, then incubation was in gas jars acclimatised at the correct temperature.

2.13 Stress application: chemical stress

2.13.1 Preparation of cells

Cells were grown to the correct growth phase and then spun down at 13,000 *g* for 5 min. The supernatant was discarded, and the cells washed in 200 µl PBS (pH 7.3). The cells were then spun at 13,000 *g* and the pellet resuspended in 200 µl PBS (pH 7.3). The cells were then diluted into temperature acclimatised 10 ml volumes of either Brucella broth or PBS (pH 7.3) to a final density of 10^8 to 10^9 cfu/ml.

2.13.2 Stress application

Additions of reagents were made to give the following final concentrations in 10 ml of medium: 0.5 mM H_2O_2 to both broth and PBS, or 4.6 mM TSP to PBS and 46 mM TSP to broth. These concentrations were known to produce a 3-log decrease in plate counts after 30 min at 20°C. Similarly hypochlorite, osmotic and pH stress conditions were imposed respectively by the addition of 0.13 mM NaOCl, 684 mM

NaCl, and acetic acid to pH 3.8. For some experiments, amiloride (0.1 mg/ml), CCCP (0.1 mM), or chloramphenicol (25 μg/ml) were added to either broth or PBS.

Ultra-violet irradiation was administered at a dose of 10 ergs/mm^2/s via a UV lamp. In a petridish (85 mm diameter), 20 ml PBS (pH 7.3) containing ~10^9 cfu/ml was dispensed. The cells were then subjected to UV irradiation for 45 s, with sampling by spread plating serial dilutions every 15 s.

2.14 Resuscitation of cold-shocked non-plateable cells

Cells were incubated under the appropriate conditions until the culture had entered the non-plateable state, where no colonies were obtained from 100 μl of culture (detection limit of <10 cfu/ml). From the non-plateable culture, 5 ml was plated out, providing a detection limit of <0.2 cfu/ml. Simultaneously, 200 μl of the non-plateable culture was serially diluted by 10^8-fold into tubes containing 1.8 ml of fresh Brucella-FBP broth or filter-sterilised spent medium (performed in multiples of five). The serial dilutions were incubated for 96h at elevated temperatures (37 or 42°C) under microaerobic atmosphere. In the event that residual plateable cells were present in the undiluted culture, serial dilution should mean that the higher dilutions will only contain non-plateable cells. Some non-plateable cells were incubated with chloramphenicol (100 μg/ml) or ampicillin (200 μg/ml) to prevent protein synthesis and cell division prior to resuscitation. In some cases the antibiotics were then removed by washing in either fresh or spent broth.

2.15 Characterisation of the resuscitation inhibitor

Resuscitation experiments were conducted to determine the characteristics of the inhibitor as described in Section 2.14. Resuscitation was compared in the presence

and absence of specific treatments of the spent medium. In some cases, spent medium was passed through 3- and 10-kDa centricons, before non-plateable cells were tested for their ability to resuscitate in the filtrate (comprising of only components less than the cut-off limit). Non-plateable cells were also examined for their ability to resuscitate in acidified (pH 2 for 30 min; subsequently raised to pH 7), and boiled (30 min) spent medium. In some experiments, proteinase K (100 μg/ml) was added to the spent medium for 30 min to degrade proteins.

2.15.1 Acidification of the spent medium

Concentrated HCl (25 μl) was added to 5 ml of spent medium (pH 6.74) for 30 min to acidify the medium to pH 2.0. Subsequently, 35 μl of 10 M NaOH was added to raise the pH again. The acidified spent medium was filter sterilised and then used as the resuscitation medium. Control tubes were prepared using fresh Brucella broth (pH 6.74) subjected to identical treatment.

2.15.2 Removal of high molecular weight molecules using microconcentrators

The Centricon-3® (3 kDa cut-off) and Vectaspin™ 3 (10 kDa cut-off) were used according to the manufacturers instructions, and the tubes spun at 7500 *g* and 10,000 *g* respectively for 1h at 4°C. The subsequent filtrate (comprising molecules below the mol. wt cut-off) was used as the resuscitation medium, following the protocol described in Section 2.14.

2.16 Identification of stress proteins

2.16.1 Preparation of cells and protein samples

Three 500 ml volumes of Brucella-FBP broth were inoculated with 1 ml of an overnight starter culture, and incubated microaerobically at 37°C for 16h. In order to stress the cells, 0.1 mM H_2O_2 or 32 mM TSP were added. An untreated culture was used as the control. The cultures were incubated for a further hour, before being centrifuged at 13,000 *g* in a Sorvall® RC-5B centrifuge at 4°C. The pelleted cells were then resuspended into 5 ml sonication/solubilisation solution, sonicated (three 20 s pulses at 10 s intervals). Dnase (50 μg/ml) was added and the samples stored on ice for 5 min. The samples were then spun at 13,000 *g* in a bench centrifuge for 1 min to pellet large debris. The resultant supernatant was transferred to a clean container

Sonication/solubilisation solution

2 ml 1M Tris-HCl (pH 8)
0.5 ml NP40
2.5 ml 400 mM EDTA solution
0.02 g PMSF
0.1 g $MgCl_2$
0.5 ml RNase (10 mg/ml)
to 5 ml with sterile water

The protein concentration was determined via the modified Lowry assay (Section 2.10.1). The required volume to give 10 mg protein was determined, and the proteins were then acetone precipitated (ratio of 0.2 ml protein sample per 2 ml ice-cold acetone) overnight at –20°C. The proteins were then spun

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0.5 ml NP40
2.5 ml 400 mM EDTA solution
0.02 g PMSF
0.1 g $MgCl_2$
0.5 ml RNase (10 mg/ml)
to 5 ml with sterile water

The protein concentration was determined via the modified Lowry assay (Section 2.10.1). The required volume to give 10 mg protein was determined, and the proteins were then acetone precipitated (ratio of 0.2 ml protein sample per 2 ml ice-cold acetone) overnight at –20°C. The proteins were then spun for 15 min at 13,000 *g* in a bench centrifuge, before the protein pellet was washed twice in ice-cold acetone and air-dried.

2.16.2 Two-dimensional PAGE

The protein samples were acetone precipitated to give a total protein load of 10 mg protein per gel, and resuspended into 240 µl solubilisation solution. One control gel was run for every three test gels (H_2O_2 or TSP stressed), providing a total of 30 mg protein for each stress. The first and second dimensions were run as previously described in Section 2.10.3.

Solubilisation solution

100 µl IPG buffer (pH 3-10L)
2.1 g Urea
0.76 g Thiourea
0.015 g DTT
0.2 g CHAPS
25µl Triton X-100
to 5 ml with sterile water

2.16.3 Staining the gels

The gels were rinsed three times in sterile water for 5 min periods. The gels were then stained using 50 ml GelCode® Blue Stain Reagent, with gentle shaking for 2h. Periodically the gels were checked for spot development. After 2h the staining reagent was replaced with deionised water several times over a 60 min period to enhance the sensitivity of spot detection.

The stained gels were then scanned (Hewlett Packard ScanJet 6100 C/T) into a computer file and the profiles of the stressed gels compared to the control gel using the 2D Gel Analysis Software (Phoretix, Non-Linear Dynamics Ltd). The proteins of interest were then excised, and placed into clean, clearly labelled eppendorfs and stored at -80°C until further processing.

2.16.4 Tryptic digestion of proteins

The proteins of interest were excised from triplicate gels, and identical proteins were pooled into clearly labelled eppendorfs. SDS was removed by incubation (twice) for 30 min in the presence of 300 µl 200 mM ammonium bicarbonate in 50% (v/v) acetonitrile at 30°C. The proteins were reduced in 300 µl 20 mM DTT, 200 mM ammonium bicarbonate, 50% (v/v) acetonitrile at 30°C for 60 min. The proteins were then washed, three times with 300 µl 200 mM ammonium bicarbonate, 50% (v/v) acetonitrile. The cysteine residues were alkylated by incubation in the dark with 100 µl freshly prepared 50 mM iodoacetamide, 200 mM ammonium bicarbonate, 50% (v/v) acetonitrile solution for 20 min at room temperature. The proteins were then washed (three times) with 500 µl 20 mM ammonium bicarbonate, 50% (v/v) acetonitrile solution. The bands were then chopped into 2 mm by 1 mm pieces on a clean tray and spun at 13,000 *g* in a bench centrifuge for 2 min. The gel pieces were then covered with acetonitrile until they turned white. The acetonitrile was then decanted and the gel pieces allowed to air dry. The gel pieces were swollen in 200 µl of a 50 mM ammonium bicarbonate, 5 µg/ml trypsin solution at 4°C. Once the gel pieces had swollen, they were incubated at 32°C for 16-24h.

The samples were spun for 2 min at 13,000 *g* in a bench centrifuge, before the supernatant was decanted (keep the supernatant). Then 100 µl of acetonitrile was added and the solution was incubated at room temperature for 15 min until the gel pieces turned white. The supernatant was decanted (keep supernatant) and pooled. The volume of the sample was then reduced to 100 µl using a Speed Vac Concentrator (Savant). Care was taken not to dry the sample. The protein sample was

stored at -20°C in 60-100 µl aliquots until analysis by nano-electrospray mass spectrometry.

200 mM ammonium bicarbonate/ 50% (v/v) acetonitrile

(per 100 ml)

1.58 g ammonium bicarbonate
50 ml acetonitrile
50 ml distilled water

50 mM iodoacetamide/ 200 mM ammonium bicarbonate/ 50% (v/v) acetonitrile

(per 100 ml)

0.925 g iodoacetamide
1.58 g ammonium bicarbonate
50 ml acetonitrile
50 ml distilled water

20 mM dithiothreitol/ 200 mM ammonium bicarbonate/ 50% (v/v) acetonitrile

(per 100 ml)

0.31 g dithiothreitol
1.58 g ammonium bicarbonate
50 ml acetonitrile
50 ml distilled water

20 mM ammonium bicarbonate/ 50% (v/v) acetonitrile

(per 100 ml)

0.158 g ammonium bicarbonate
50 ml acetonitrile
50 ml distilled water

50 mM ammonium bicarbonate

(per 100 ml)

0.395 g ammonium bicarbonate
100 ml distilled water

59:1 Trypsin/ 50 mM ammonium bicarbonate solution

(per 100 ml)

59 µl 50 mM ammonium bicarbonate solution
1 µl trypsin

2.16.5 Nanoelectrospray tandem mass spectrometry (Welmet)

Protein samples were desalted via passage through a reverse phase cartridge prior to running through the Thermoquest LCQ mass spectrometer (Welmet protein sequencing facility). Peptides produced a characteristic protein profile. Double-charged ions were selected for further splitting by bombardment, with the resultant peptide profile representing an amino acid sequence that was searched for homologues in a protein database (SEQUEST v. C1). Confirmation of the protein identity was performed by obtaining the full protein sequence from the National Centre for Biotechnology (NCBI; http://www.ncbi.nim.nih.gov) and performing artificial tryptic digestion using the Mascot software (http://www.matrixscience.com). The molecular weights of the single peptide peaks from the initial protein profile were identified from the artificial tryptic digestion, confirming the presence of these peptides within the protein, and thus the identification of the protein.

Section 3

Effectiveness of activity dyes

3.0 Results and discussion: Effectiveness of activity dyes

Various dyes were examined for their ability to demonstrate maintenance of metabolic activity or structural integrity in non-plateable cells. Dyes studied included calcofluor white, propidium iodide, INT, rhodamine 123 and carboxyfluorescein diacetate.

3.1 Calcofluor white

3.1.1 Effect of calcofluor white on plateable and boiled *Campylobacter jejuni* 81116 cells

Exponential phase plateable and boiled (30 mins) cells were incubated in the presence of 5 μM CFW for 5 min at 37°C, and the percentage of stained cells determined. Boiled (dead) cells preferentially stained with CFW (Table 3.1), demonstrating the potential of CFW to be used as an indicator of 'activity' in *C. jejuni*, as previously shown in other bacteria (Mason *et al.*, 1995). Hence CFW stains cells with impaired activity.

Treatment	Plate count (0 min; cfu/ml)	Plate count (30 min; cfu/ml)	CFW-stained (%)	Alive/ dead?
Plateable	2.46×10^9	2.48×10^9	1.59 ± 0.6	Alive
Boiled (30 mins)	2.35×10^9	0	99.93 ± 0.18	Dead

Table 3.1: Effect of CFW-staining on live and boiled *Campylobacter jejuni* 81116 cells

3.1.2 Effect of CFW concentration, incubation time, and incubation temperature on the staining of plateable and boiled cells

Live (plateable) and boiled cells were stained for 5 min in the presence of 0-100 μM CFW at 37°C (Table 3.2). The plate and total counts were determined as $2.48 \pm 0.39 \times 10^9$ cfu/ml and $2.71 \pm 0.25 \times 10^9$ cells/ml respectively.

CFW concentration (μM)	CFW-stained cells (%)		Calculated inactive cells (cells/ml)	
	Plateable	Boiled	Plateable	Boiled
0	0	0	0	0
0.5	0	3.9 ± 3.7	0	1.1×10^8
1	0	96 ± 2.1	0	2.6×10^9
5	0.025 ± 0.014	99.8 ± 0.3	6.8×10^5	2.7×10^9
10	17.2 ± 8.6	100 ± 0 †	4.8×10^8	2.71×10^9
50	90.3 ± 4.5	100 ± 0 †	2.44×10^9	2.71×10^9
100	100 ± 0	100 ± 0 †	2.71×10^9	2.71×10^9

Table 3.2: Effect of CFW concentration on the percentage of stained *Campylobacter jejuni* 81116 cells. † = high non-specific background. Percentage inactive cells are calculated from the total count data and the % CFW-stained data, for comparison with the plate counts.

As the CFW concentration increases, the percentage of stained plateable and boiled cells increased. Concentrations above 5 μM overestimated the number of inactive/dead cells in the plateable suspension, whilst 0.5 μM CFW underestimated the number of inactive/dead cells in the boiled suspension. Therefore a CFW concentration of 5 μM proved optimal for the assay.

Incubation time or temperature did not significantly affect the percentage of cells stained by CFW over a 30 min period (Table 3.3). Therefore a 5 min incubation period at 37°C was chosen for subsequent CFW assays.

Incubation time (min) §	CFW-stained cells (%)		Temperature (°C)	CFW-stained cells (%)	
	Plateable	Boiled		Plateable	Boiled
5	1.1 ± 0.7	99.5 ± 0.7	4	3.5 ± 1.5	100 ± 0
10	1.5 ± 0.6	99.6 ± 0.2	20	3.8 ± 2.4	99.9 ± 0.2
30	2.9 ± 2.4	99.9 ± 0.25	37	3.8 ± 2.0	99.9 ± 0.35

Table 3.3: Effect of incubation time and temperature on CFW staining in *Campylobacter jejuni* 81116. § = All samples were incubated at 37°C

3.1.3 Determination of the mechanism of action of CFW

Live (plateable) and boiled cells of the Gram negative species, *C. jejuni*, *E. coli*, *S. typhimurium*, *Klebsiella*, and the Gram positive species, *B. subtilis* and *S. aureus* were incubated for 5 mins with 5 μM CFW at room temperature (Table 3.4).

Bacterial species	Gram stain (+/-)	CFW-stained cells (%)	
		Plateable	Boiled
C. jejuni 81116	-	1.1 ± 0.8	100 ± 0
E. coli B	-	2.4 ± 1.6	100 ± 0
S. typhimurium LT5	-	3.1 ± 1.3	100 ± 0
Klebsiella spp.	-	4.4 ± 2.6	100 ± 0
B. subtilis W168	+	100 ± 0 †	100 ± 0
S. aureus	+	100 ± 0 †	100 ± 0

Table 3.4: Effect of CFW on plateable and boiled cells of Gram negative and Gram positive bacteria. † = dull fluorescence

Gram negative bacteria displayed differential CFW staining between plateable and boiled (dead) cells. Boiled cells were preferentially stained, whilst plateable cells excluded CFW. In contrast, both plateable and boiled Gram positive cells stained with CFW, although the fluorescence intensity was reduced in plateable cells. This indicates that CFW exclusion in live (plateable) Gram negative cells is perhaps due to cell wall differences between Gram negative and Gram positive bacteria. It is known that CFW binds to β(1-3) and β(1-4) glycosidic linkages (Maeda & Ishida, 1967), which are present in peptidoglycan (Hammond *et al.*, 1984). Assuming that peptidoglycan (PG) is the binding site, CFW would have access to the PG of Gram positive bacteria, however access to Gram negative PG would be prevented by the outer membrane. Boiling cells would result in perturbation of outer membrane structure, permitting CFW access to the PG. It is assumed that the porins have small exclusion limits, preventing access of CFW (M_r = 941) to the PG. It is known that the

major porins in *E. coli* (OmpC and OmpF) have mol. wt cut-offs of ~600-700 Da (Nikaido, 1994), so it is unlikely that CFW would readily diffuse through the outer membrane via the porins. LPS 'O' specific side-chains also possess β(1-3) and β(1-4) glycosidic linkages (Hammond, *et al.*, 1984), however, live plateable cells do not stain with CFW, indicating that LPS is not the site of binding. It is proposed that outer membrane blockage is the main mode of activity, preventing CFW staining in live (plateable) Gram-negative cells.

3.1.4 Toxicity of calcofluor white to *Campylobacter jejuni* 81116 and *Escherichia coli* B cells

A range of CFW concentrations from 0-100 mM were examined for toxicity to *C. jejuni* and *E. coli* cells (Figure 3.1). Neither bacterial species showed any adverse affects to the highest CFW concentration (100 mM), with growth rates identical to the control, hence it is concluded that the concentration used in the CFW assay (5 μM) would not have any deleterious effects on the cells. This is in accordance with results in the literature indicating lack of toxicity in *E. coli* and *B. subtilis* (Darken, 1962).

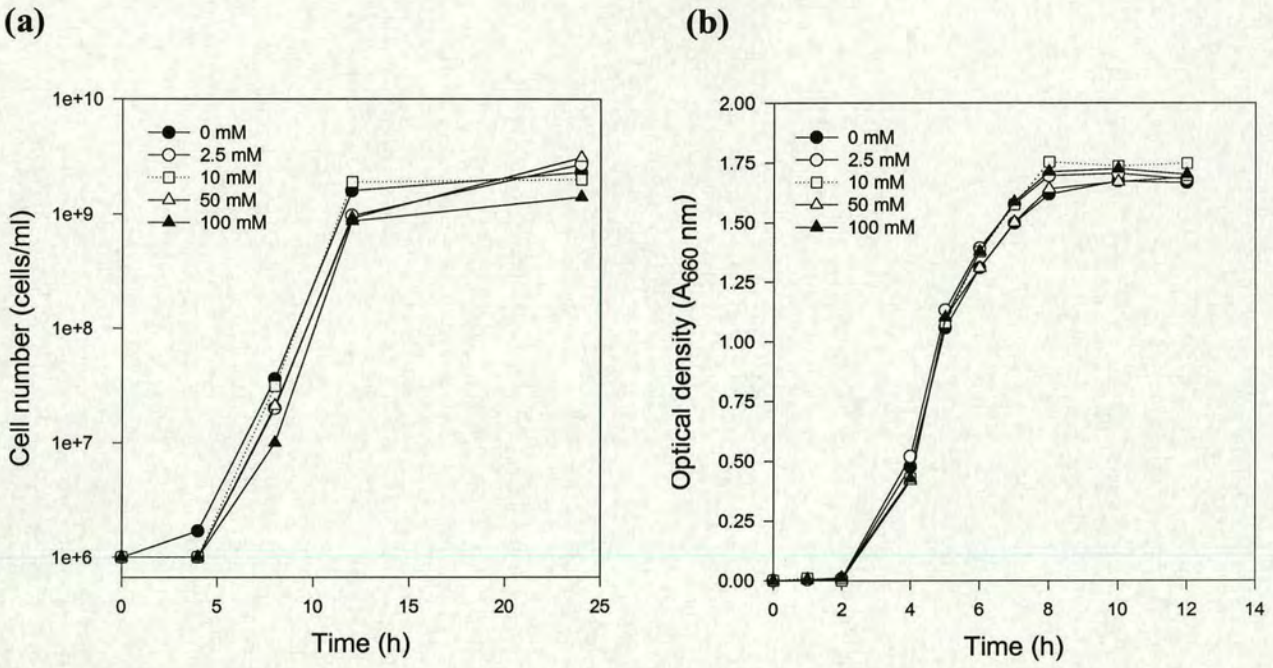


Figure 3.1: Effect of CFW concentration on the growth of (a) *Campylobacter jejuni* 81116 and (b) *Escherichia coli* B cells

3.1.5 Effect of sphaeroplast formation on CFW staining

Sphaeroplasts were formed according to the method of Birdsell & Cota-Robles, 1967, with a slight modification for *C. jejuni* cells, where a lysozyme concentration of 200 μg/ml was used (Moran & Upton, 1986). The kinetics of sphaeroplast formation in *C. jejuni* 81116 and *E. coli* B are depicted in Figure 3.2.

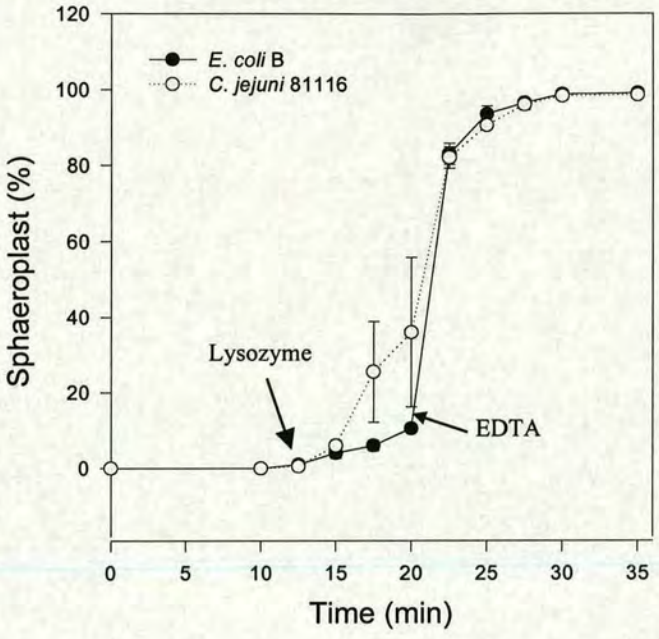


Figure 3.2: Kinetics of sphaeroplast formation in *Escherichia coli* B and *Campylobacter jejuni* 81116 cells

Sphaeroplasts were examined for their ability to stain with CFW in the presence and absence of various treatments (Table 3.5). As observed in normal cells, untreated sphaeroplasts deemed to be alive displayed the ability to exclude CFW, whilst boiled sphaeroplasts (dead) lost this ability. This occurred despite the removal of the outer membrane and substantial reduction in PG during sphaeroplast formation, perhaps indicating alternative CFW binding sites. Interestingly, sphaeroplasts incubated in the presence of CCCP (pmf uncoupler), had reduced ability to exclude CFW observed in untreated sphaeroplasts (Table 3.5), substantiating the conclusions of Mason *et al.*, 1995, that CFW stains cells with compromised membrane potentials. Efflux pumps extruding CCCP could account for the discrepancy between CCCP-treated cells and boiled cells. It is known that *E. coli* possesses such extrusions pumps like EmrAB and AcrAB (Paulsen *et al.*, 1996), and *C. jejuni* also possesses homologues of AcrAB and other efflux systems (Section 7.8).

Species	Untreated	Boiled (2 min)	CCCP (1 μM)
C. jejuni 81116	1.4 ± 0.6	100 ± 0	52 ± 5.8
E. coli B	2.3 ± 1.3	89 ± 4.5	16 ± 3.1

Table 3.5: Effect of various treatments on the CFW staining ability of *Campylobacter jejuni* 81116 and *Escherichia coli* B sphaeroplasts

In view of the barrier imposed by the outer membrane permeability to CFW (Table 3.4), it is unlikely that the membrane potential plays a large part in CFW exclusion when the cells have an intact outer membrane. Nevertheless, any treatment that disrupts the permeability of the outer membrane will permit CFW access to its PG and intracellular binding sites (see Section 3.5). Hence, CFW excluding ability is primarily due to possession of an intact outer membrane.

3.2 INT as an indicator of respiratory dehydrogenase activity

3.2.1 Effect of plateable and boiled cells on INT reduction

INT is reduced by active respiratory dehydrogenases (Thom *et al.*, 1993), reportedly indicating cellular respiration in bacteria (Betts *et al.*, 1989). Exponential phase cells were boiled (30 mins) and incubated in the presence of 1 mM INT for 10 min at 37°C, and the percentage of INT-reducing cells determined (Table 3.6). It was observed that INT-reducing capacity correlates with the cells possessing plating ability, as observed in other bacteria (Betts *et al.*, 1989).

Treatment	Plate count (cfu/ml)	Total count (cells/ml)	INT-reducing cells (%)
Plateable	3.3×10^9	3.41×10^9	99.9 ± 0.15
Boiled (30 min)	0	1.6×10^9	0.7 ± 0.2

Table 3.6: Effect of boiling *Campylobacter jejuni* 81116 cells for 30 min on INT-reducing capacity

3.2.2 Effect of INT concentration, incubation time, temperature and succinate on the INT reducing capacity of plateable and boiled cells.

Exponential phase (12h) cells were incubated in the presence of 0-10 mM INT for 30 min at 37°C, and the resultant INT-reducing cells expressed as a percentage of the total cell population (Table 3.7). INT concentrations of 5-10 mM produced high non-specific background, as well as false positives in the boiled suspension, whilst 0.05-0.1 mM INT did not stain all 'active' cells as indicated by the lack of correlation with the plate counts. Therefore, 0.5 mM INT provided the closest correlation between the calculated 'active' counts and the plate counts.

INT concentration (mM)	INT-reducing cells (%)		Calculated active cells (%)	
	Plateable	Boiled	Plateable	Boiled
0	0 ± 0	0 ± 0	0	0
0.05	87.4 ± 3.3	0.31 ± 0.2	3×10^9	5×10^6
0.1	95.7 ± 0.7	3.1 ± 2.1	3.26×10^9	5×10^7
0.5	98.8 ± 1.1	1.86 ± 1.0	3.3×10^9	2.9×10^7
1.0	99.4 ± 1.1	4.52 ± 2.1	3.4×10^9	7.2×10^7
5.0	100 ± 0 †	10.9 ± 2.3	3.41×10^9	1.7×10^8
10.0	100 ± 0 †	12.7 ± 6.0	3.41×10^9	2×10^8

Table 3.7: Effect of INT concentration on the percentage of INT-reducing plateable and boiled cells. † = high non-specific background. Plate counts and total counts for plateable cells were 3.3 $\times 10^9$ cfu/ml and 3.41 $\times 10^9$ cells/ml respectively. Plate counts and total counts for boiled cell were 0 cfu/ml and 1.6 $\times 10^9$ cells/ml respectively.

Exponential phase (12h) cells were incubated in the presence of 0.5 mM INT for 1-60 mins at 37°C or for 30 mins at temperatures ranging from 4 to 37°C, and the resultant INT-reducing cells expressed as a percentage of the total population (Table 3.8). High non-specific background was obtained after a 30-60 min incubation period, and lower periods (1-5 min) underestimated the number of plateable cells. Therefore 10 min was chosen as the incubation period for the INT assay. Temperature did not significantly affect the percentage of population reducing INT, however, the rate of

INT reduction increased with temperature, as indicated by larger formazan inclusions at 37°C compared to 4°C. Therefore, the INT assay will use an incubation temperature of 37°C for consistency with the other enzyme based assay, using CFDA (Section 3.4.3).

Incubation time (min)	INT-reducing cells (%)		Temperature (°C)	INT-reducing cells (%)	
	Plateable	Boiled		Plateable	Boiled
1	74.3 ± 4.1	0.4 ± 0.3	4	99.2 ± 0.97	3.1 ± 1.6
5	91.4 ± 2.8	1.8 ± 1.2	20	99.5 ± 0.44	2.6 ± 1.4
10	99.2 ± 0.5	3.2 ± 2.3	37	99.8 ± 0.45	2.8 ± 2.1
30	99.8 ± 0.4†	8.3 ± 2.8			
60	99.7 ± 0.3†	12.0 ± 2.6			

Table 3.8: Effect of incubation time and temperature on the percentage of INT-reducing plateable and boiled cells. Plate counts and total counts for plateable cells were 3.3×10^9 cfu/ml and 3.41×10^9 cells/ml respectively. Plate counts and total counts for boiled cell were 0 cfu/ml and 1.6×10^9 cells/ml respectively. † = high non-specific background

Exponential phase (12h) cells were incubated in the presence of 0.5 mM INT and 0-10 mM succinate for 10 min at 37°C, and the resultant INT-reducing cells expressed as a percentage of the total cell population (Table 3.9). Succinate is a substrate for respiratory dehydrogenases, and acts as a stimulator of respiratory activity (Boucher *et al.*, 1994).

Succinate concentration (mM)	INT-reducing cells (%)		INT-reducing cells (cells/ml)	
	Plateable	Boiled	Plateable	Boiled
0	100 ± 0	0 ± 0	2.83×10^9	0
0.5	100 ± 0	2.4 ± 0.8	2.83×10^9	4.2×10^7
1	96.6 ± 0.6	11.7 ± 3.9	2.73×10^9	2×10^8
5	98.3 ± 0.4	18.7 ± 4.3	2.81×10^9	3.3×10^8
10	97.8 ± 0.3	25.1 ± 4.2	2.77×10^9	4.4×10^8

Table 3.9: Effect of succinate concentration on the percentage of INT-reducing plateable and boiled cells. Plate count and total counts for plateable cells were 2.6×10^9 cfu/ml and 2.83×10^9 cells/ml respectively. Plate count and total counts for boiled cells were 0 cfu/ml and 1.75×10^9 cells/ml respectively.

Increased succinate concentrations had no effect on the INT-reducing capacity of plateable cells, but significantly increased the capacity of boiled cells to reduce INT, despite no plateable cells being present, indicating that a percentage of boiled cells were injured or that the enzyme(s) responsible for INT-reduction are heat-stable or can renature. Therefore such enzymes would be capable of activity within presumably dead cells (boiled for 30 min). A succinate concentration of 1 mM is optimal for the INT assay because it permits maximal staining of plateable cells, and indicates INT-reducing ability in non-plateable or dead cells. It is proposed that two samples should be performed, with and without 1 mM succinate. This allows detection of cells which require succinate to induce INT-reducing capacity.

In this text, non-plateable cells that can reduce INT will be referred to as possessing INT-reducing activity (equivalent to ABNC) rather than being in a 'VBNC' state. There is some doubt as to why cells boiled for 30 min (presumably dead) should still reduce INT. The possibility that such cells are injured was examined, but no plateable cells were recovered over 7 days incubation at 37°C. This implies that the enzyme responsible for INT-reduction can withstand boiling for 30 min in dead cells, supporting the hypothesis that under certain conditions non-plateable cells may be no more than bags of (active) enzymes.

3.3 Potential of propidium iodide as a metabolic dye

A variety of physiological forms of *C. jejuni* were examined for their ability to exclude propidium iodide (PI), indicative of cytoplasmic membrane integrity. Cells were incubated with 5 μg/ml PI for 10 mins at 37°C (Table 3.10).

Sample	Morphology	Plate count (cfu/ml)	PI-stained cells (%)
Exponential phase cells (12h)	spiral	1.23×10^9	0
Boiled (30 min)	spiral	<10	100 [B]
Aerated (20d at 37°C)	coccoid	<10	100 [L]
Aerated (9d at 4°C)	spiral	1.87×10^3	0

Table 3.10: Effect of the physiological state of *Campylobacter jejuni* 81116 cells on propidium iodide exclusion. B = bright fluorescence and L = low fluorescence

Plateable cells did not stain with PI, indicating the presence of an intact cell membrane. In contrast, boiled cells stained brightly with PI, indicating access to nucleic acid binding sites, and thus loss of membrane integrity. Coccoid cells formed via prolonged aeration, stained with PI, but the fluorescence was markedly reduced compare to boiled cells, indicating that these coccoid cells possess impaired membrane integrity, consistent with published reports (Buck *et al.*, 1983 and Jones *et al.*, 1991a). The reduced fluorescence could be due to loss of nucleic acids either by degradation or leakage observed in coccoid forms (Moran & Upton, 1986). Aeration at 4°C resulted in a heterogeneous population of plateable and non-plateable spiral cells, however all possessed membrane integrity as indicated by lack of PI staining. In conclusion, as has been reported for other bacteria (López-Amorós *et al.*, 1995), PI inversely correlates with plate counts, and can be used as an indicator of membrane integrity in *C. jejuni*.

3.4 Use of 6-carboxyfluorescein diacetate as an indicator of esterase activity

3.4.1 Effect of buffer pH on autohydrolysis of CFDA

Buffers were constructed providing a pH range from 4-9. In the absence of cells, the solutions were incubated for 60 min at 37°C with 1 μM CFDA, and the solution colour and fluorescence intensity noted (Table 3.11).

Buffer pH	Solution colour	Fluorescence intensity (513 nm)
4.0	colourless	23.7
6.0	colourless	43.5
6.5	colourless	51.5
7.0	very light yellow	271.6
7.6	light yellow	583.4
9.0	bright yellow	999.9

Table 3.11: Effect of buffer pH on the autohydrolytic action of CFDA

As the buffer pH was increased the rate of autohydrolysis of CFDA to carboxyfluorescein increased. This phenomenon has been documented previously for other fluorogenic substrates (Haughland, 1999). The CFDA assay will use a pH 6.5 phosphate buffer, as negligible autohydrolysis was observed.

3.4.2 Potential of CFDA as a metabolic stain

Plateable and boiled cells were incubated in phosphate buffer (pH 6.5) for 90 min with 1 μM CFDA at 37°C. CFDA cleavage by esterase activity was monitored by spectrofluorometry and microscopy (Table 3.12). Spectrofluorimetry detects the cleavage product (carboxyfluorescein) present in the extracellular medium, whilst microscopy detects carboxyfluorescein within the cells.

Sample	Plate count (log cfu/ml)	Total count (log cells/ml)	CFDA-stained cells (%)	Fluorescence (513 nm)
Plateable	9.38 ± 0.13	9.45 ± 0.24	4.6 ± 2.5	116.4 ± 12.1
Boiled (30 min)	0	9.38 ± 0.26	0 ± 0	13.4 ± 2.6

Table 3.12: Effect of plateable and boiled *Campylobacter jejuni* 81116 cells on CFDA cleavage

Fluorescent microscopy was unsatisfactory because it indicated that a small percentage (4.6%) of cells possessed esterase activity, despite 86% of the cells being plateable and the colour of the solution being bright yellow (indicative of CFDA

cleavage). The spectrofluorimetry data indicates that plateable cells possess esterase activity whilst boiled cells do not. It appears that the cleaved fluorescent product is actively pumped into the surrounding medium, hence the difference between the microscopic and spectrofluorimetric methods. Therefore the spectrofluorometric assay will be used for the detection of esterase activity. It is evident that CFDA will only give a qualitative indication of esterase activity within the population of cells. This reduces the effectiveness of CFDA as an indicator of metabolic activity.

3.4.3 Effect of CFDA concentration, incubation temperature, and incubation time on esterase activity in *Campylobacter jejuni* 81116

Exponential phase cells (10^8 cells/ml) were incubated for 2h at 37°C in the presence of CFDA concentrations ranging from 0-20 μM CFDA (Figure 3.3a), for 2h in the presence of 1 μM CFDA at incubation temperatures ranging from 4 to 37°C (Figure 3.3b), or for periods ranging from 0-4h in the presence of 1 μM CFDA at 37°C (Figure 3.3c).

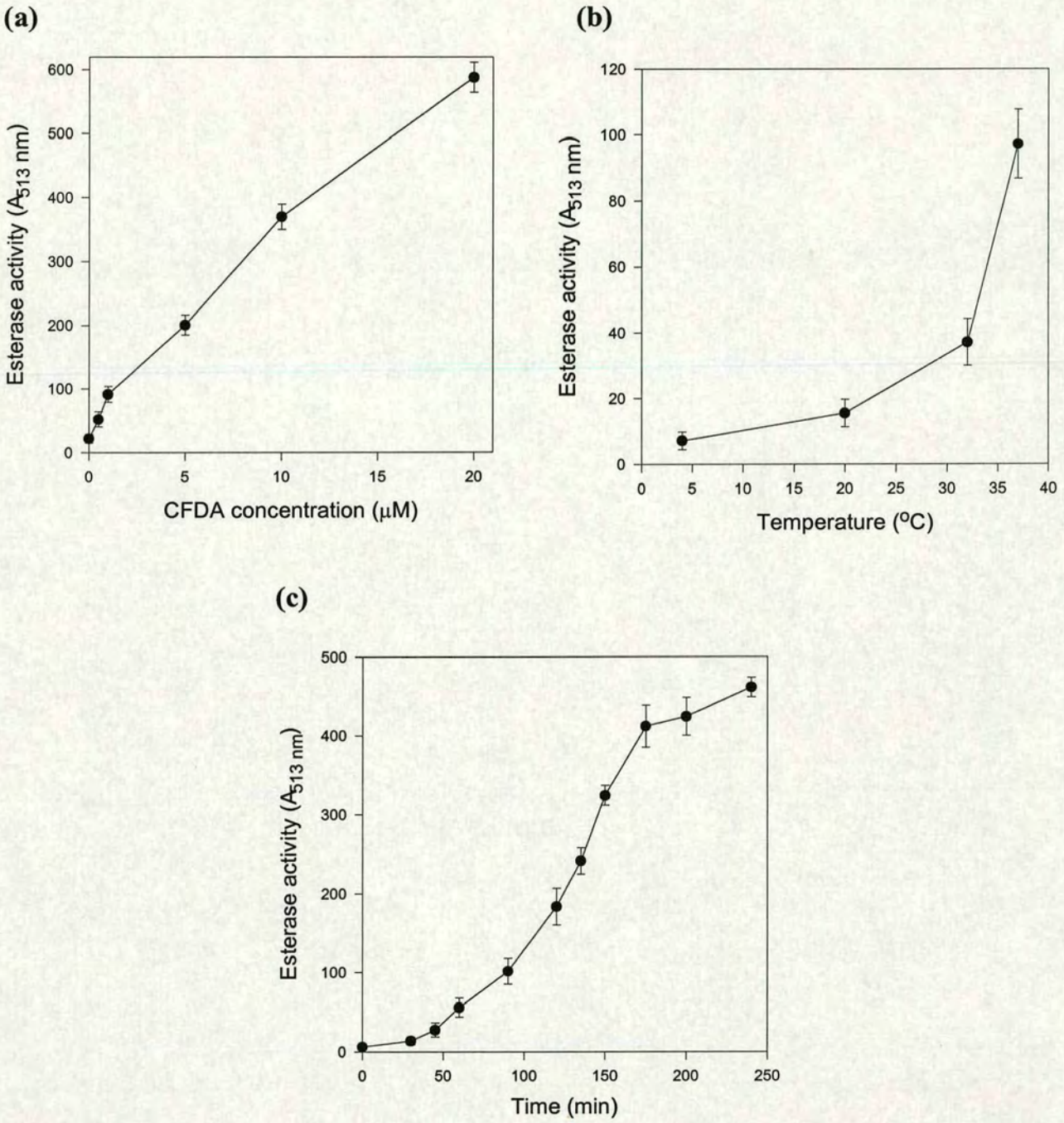


Figure 3.3: Effect of (a) CFDA concentration, (b) incubation temperature, and (c) incubation time on *Campylobacter jejuni* 81116 non-specific esterase activity

The esterase enzyme probably obeys Michaelis-Menten kinetics. As the CFDA concentration increases (substrate) the esterase activity (enzyme) increases

(Figure 3.3a). The velocity of the reaction would plateau as eventually the active sites of the enzyme are saturated. The Michaelis constant (K_m) for the esterase enzyme is greater than 10 μM because the maximum velocity (V_{max}) of the reaction had not occurred after incubation with 20 μM CFDA for 90 min (Figure 3.3a). In the assay, 5 μM CFDA will be used because it provides a reasonable intensity reading after 90 min incubation. The esterase activity increases as the temperature increases (Figure 3.3b), as expected for an enzyme reaction. Hence 37°C will be used as the temperature for the CFDA assay. As the incubation time increases, initially a logarithmic increase in CFDA cleavage is observed (50-120 min), followed by a decreased rate of synthesis as the substrate becomes limited after 150 min, producing a sigmoidal curve (Figure 3.3c). It is proposed that the CFDA assay should be performed over 90 min, providing a maximum fluorescence intensity of ~200, permitting changes in fluorescence to be observed (the detectable intensity limit of the spectrofluorometer is 999.9).

3.4.4 Effect of cell density on CFDA hydrolysis

Exponential phase cells were serially diluted into phosphate buffer (pH 6.5), and the plate counts determined. The diluted cells were incubated with 1 μM CFDA at 37°C for 90 min (Table 3.13).

Cell density (cfu/ml)	Esterase activity ($A_{513\ nm}$)	Increase in activity (-fold)
4.5×10^9	468.3 ± 38.4	6.7
2.4×10^8	69.6 ± 11.2	1.8
2.0×10^7	38.4 ± 5.1	1.9
3.5×10^6	20.1 ± 3.7	-

Table 3.13: Effect of cell density on *Campylobacter jejuni* 81116 esterase activity

An approximate doubling in esterase activity was observed as the cell density increased by ~1 log unit. However a 6.7-fold increase in esterase activity was observed as the density increased from 10^8 to 10^9 cfu/ml. Therefore for the use of CFDA as a metabolic stain, it is important for equivalent densities to be used for comparison. For non-growth conditions (i.e. 4°C) the cell density should not change over the course of experimentation, hence CFDA can be used as an indicator of activity in such circumstances.

3.5 Comparison of metabolic dyes and inhibitors in *Escherichia coli* B and *Campylobacter jejuni* 81116

The effect of sodium azide, CCCP, polymyxin B, and boiling on the staining ability of *E. coli* B (Table 3.14) and *C. jejuni* 81116 (Table 3.15) cells was examined microscopically.

		Stained cells (%)				
Stain	Plateable	Boiled (30 min)	Sodium azide (50 mM)	CCCP (5 μM)	Polymyxin B (20 μg/ml)	EDTA (100 Mm)
CFW	2^{L}	100^{B}	1^{B}	100^{B}	100^{L}	100^{B}
Rh123	100^{B}	100^{B}	100^{B}	0^{L}	100^{B}	100^{B}
INT	100	0	40	100	100	100
AO	$100^{L\dagger}$	100^{B}	80^{B}	100^{B}	100^{B}	100^{B}

Table 3.14: Effect of various treatments on the action of metabolic dyes in *Escherichia coli* B. L = low fluorescence and B = bright fluorescence. † = Plateable cells appeared to 'pump' acridine orange out of the cells becoming almost non-fluorescent within 30 secs. Mean of three separate experiments

Stain	Stained cells (%)					
	Plateable (untreated)	Boiled (30 min)	Sodium azide (50 mM)	CCCP (5 μM)	Polymyxin B (20 μg/ml)	EDTA (100 mM)
CFW	3.6 ^{L}	100 ^{B}	10 ^{B}	100 ^{B}	100 ^{L}	86 ^{B}
Rh123	100 ^{L}	100 ^{L}	100 ^{L}	0	0	100^{B}
INT	100	0	10	100	100	100
AO	100 ^{B†}	100 ^{B}	100 ^{B}	100 ^{B}	100 ^{B}	100 ^{B}

Table 3.15: Effect of various treatments on the action of metabolic dyes in *Campylobacter jejuni* 81116. L = low fluorescence and B = bright fluorescence. † = Plateable cells appeared to 'pump' acridine orange out of the cells becoming almost non-fluorescent within 30 s. Mean of three separate experiments

Addition of sodium azide impaired respiratory activity, as indicated by a reduction in INT-reducing capacity in both *E. coli* and *C. jejuni* cells. The presence of some cells reducing INT could be due to resistant cells, or INT-reduction by respiratory enzymes unaffected by azide. Dissipation of the pmf with CCCP caused a decrease in Rh123 fluorescence compared to untreated cells, however, this was not reproducible, and fluorescence intensity differed, perhaps due to removal of CCCP by efflux pumps (Paulsen *et al.*, 1996). Hence Rh123 was discarded as a viability stain. Many researchers use flow cytometry in conjunction with Rh123 (Diaper & Edwards, 1994), and have found this useful as an indicator of viability, however others reported that oxonol dyes give a sharper distinction between live and boiled or gramicidin treated cells cells (Mason *et al.*, 1995). Perhaps flow cytometry would detect small changes in fluorescence better than microscopy.

EDTA and polymyxin B both perturb the outer membrane (Nikaido & Vaara, 1985), resulting in increased CFW staining compared to untreated cells. EDTA chelates Mg^{2+} ion, propagating LPS removal from the outer membrane, but does not kill the cells (Hammond *et al.*, 1984), therefore supporting the hypothesis that CFW binds to PG. Polymyxin punches holes in the outer and cytoplasmic membranes, and is bactericidal to both growing and non-growing cells, therefore supporting the

observations that outer membrane damage, and cytoplasmic damage will give rise to increased CFW staining (Section 3.1).

Under all treatments, cells stained bright orange with AO indicating its inappropriateness as an indicator of activity. Interestingly, plateable cells lost fluorescence after ~30 secs, perhaps due to the action of efflux pumps e.g. AcrAB which is known to remove acriflavine, a homologue of acridine orange (Paulsen *et al.*, 1996), and is present within the *C. jejuni* genome (Section 7.8).

3.6 Summary

A variety of dyes demonstrating various cellular functions have been examined in *C. jejuni* for their ability to be used as indicators of metabolic activity. In all cases, there was differential staining between plateable (live) and boiled (dead) cells. CFW staining indicates loss of outer membrane integrity. PI staining indicates loss of cytoplasmic integrity. Reduction of INT and hydrolysis of CFDA indicate possession of dehydrogenase and esterase activity respectively. Rh123 proved ineffective as an indicator of an active membrane potential under the conditions used in this study.

Section 4

Growth characteristics of *Campylobacter jejuni*

4.0 Results and discussion: Growth characteristics of *Campylobacter jejuni*

4.1 Culture of *Campylobacter jejuni* under microaerobic atmosphere at 37°C in Brucella-FBP broth

Growth of *Campylobacter jejuni* 81116 in Brucella-FBP broth was followed to determine the growth pattern in batch culture (Figure 4.1). The growth curve consisted of an exponential phase (0-14h), where the cells were morphologically short spirals, and multiplied with a doubling rate of 47.6 ± 2.6 min. Entry into stationary phase (15-42h) corresponded with elongation of the cells. During the decline phase (42-72h) the decline rate was 1.3 ± 0.28h, and there was a heterogeneous population of short spirals, filaments, and coccoid cells. The percentage of coccoid cells gradually increased until they were the dominant morphological form; however at no stage did any form completely displace another. Coccoid formation correlated with a decrease in plate counts and an increase in the percentage of cells staining with CFW (Figure 4.1). The plate counts declined to 10^3 cfu/ml after 72h, before increasing again to 10^6 cfu/ml within 24h (increase phase). During this period, coccoid cells were the predominant species, but a small population of spirals (short or filaments) existed (0.7 ± 0.12%), possibly accounting for the increase in numbers of viable cells. The presence of plateable cells, masked any putative resuscitation of coccoid cells, as observed in other studies (Bovill & Mackey, 1997). The morphological transitions observed correspond with previously published results (Griffiths, 1993 and Thomas *et al.*, 1999b; Figure 4.41-4.44). Survival of *C. jejuni* 81116 occurred deep into the decline phase (Table 4.1), for at least 206 days with the culture being predominantly coccoid in morphology. During this time the cells were probably utilising products released from lysed cells (cryptic growth).

Interestingly, optical density measurements did not correlate with the increases in plate counts. Optical density measurements approximately doubled during exponential phase, before increasing dramatically upon entry into stationary phase, correlating with formation of the elongated phenotype and a concomitant increase in cell biomass (Figure 4.1). Hence optical density measurements, as used by Rollins, *et al.*, 1983, are not a reliable determinant of growth rate in *C. jejuni*. It is proposed that plate counts should be used for calculating growth rates. Upon entry into the decline phase, optical density measurements fluctuated, probably resulting from the tendency of coccoid cells to clump (Figure 4.44).

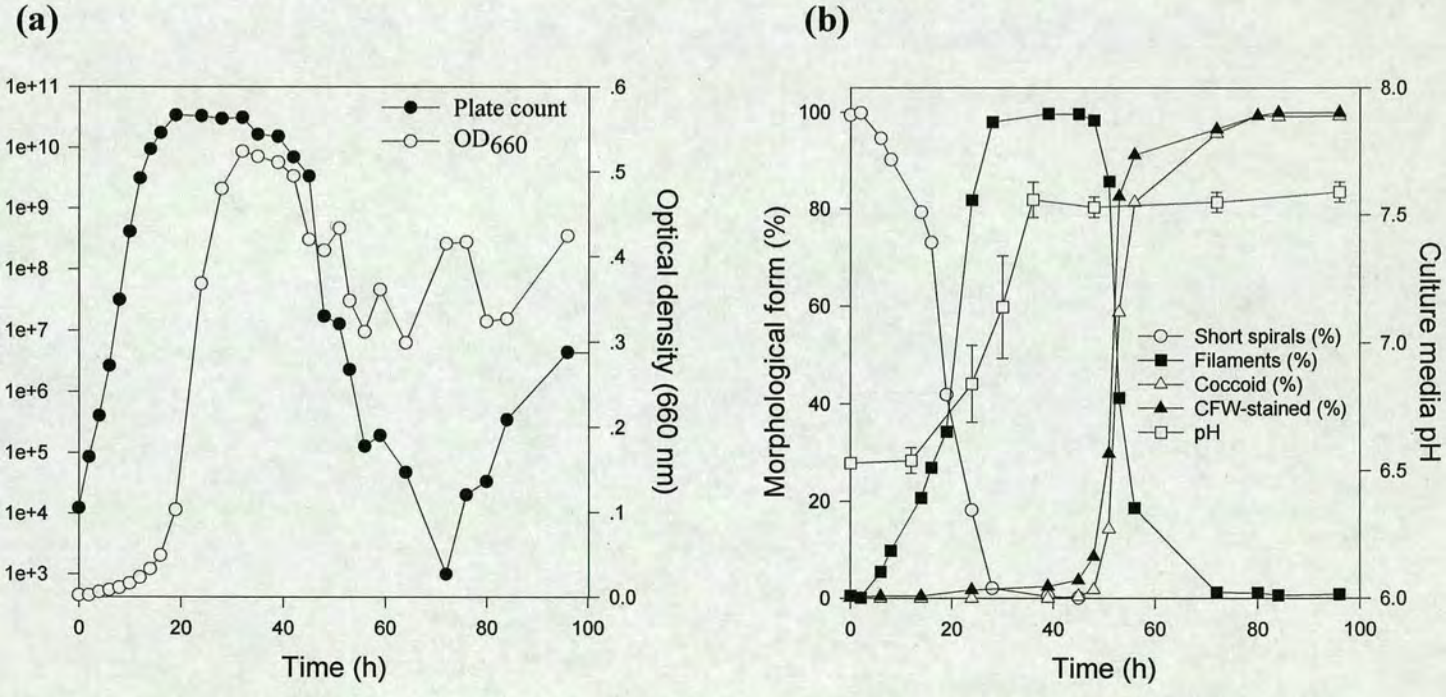


Figure 4.1: Effect of microaerobic growth of *Campylobacter jejuni* 81116 on (a) plate counts and optical density, and (b) cellular morphology and culture media pH. Mean of two separate experiments

	Incubation time (days)								
	18	27	45	126	150	177	206	267	360
Plate count (cfu/ml)	2.2×10^5	1.7×10^6	2.3×10^5	3.7×10^4	5.3×10^3	4.5×10^4	1.6×10^2	<10	<10
Coccoid (%)	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99

Table 4.1: Growth and morphology of a *Campylobacter jejuni* 81116 culture incubated for prolonged periods

Changes in the pH of the culture medium occurred during early to mid stationary phase, correlating with cell elongation (Figure 4.1). Such increases in pH during the culture of campylobacters (Rollins *et al.*, 1983) and *H. pylori* (Bode *et al.*, 1993) have been previously noted, perhaps due to the action of deaminases whose end-products are basic. Deaminases specific for asparagine, aspartate, serine, glutamate and glutamine have been observed in *C. jejuni* (Karmali *et al.*, 1984 and Leach *et al.*, 1997). Whether the expression or activity of these deaminases is growth phase dependent is unknown. The possibility that the increase in external pH due to metabolism resulted in elongation or coccoid formation was examined by observing morphological changes upon growth in Brucella-FBP broth constructed in the presence and absence of phosphate buffer (pH 6.5) (Figure 4.2).

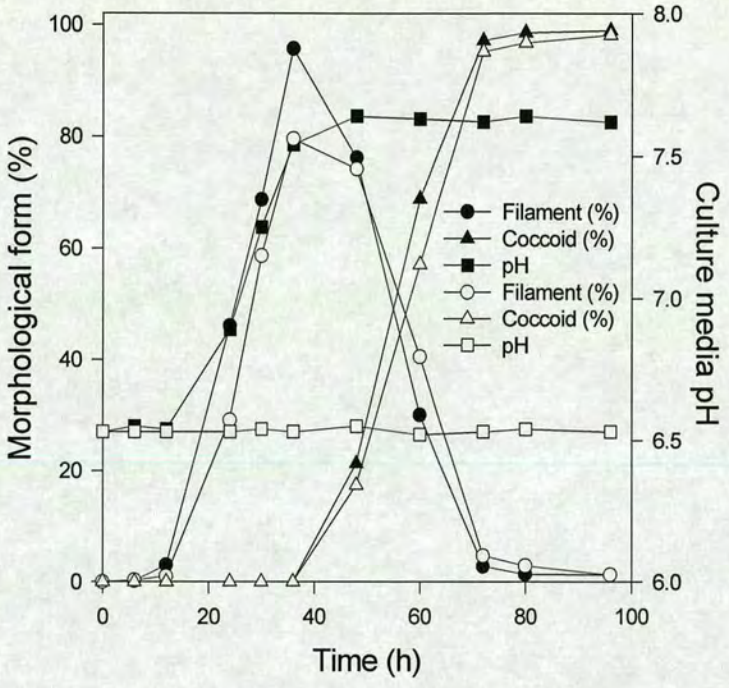


Figure 4.2: Effect of buffering the culture medium on morphological changes observed during growth of *Campylobacter jejuni* 81116. Closed symbols = Brucella-FBP broth; open symbols = phosphate buffered [pH 6.5] Brucella-FBP broth. Mean of two separate experiments.

Buffering the medium prevented the increase in culture media pH. However, no differences were observed in the rate of formation of elongated or coccoid cells, indicating that the rise in pH was not the cause of these morphological forms. However, this does not exclude the possibility that elongation resulted in the pH increase, perhaps due to increased deaminase production.

Additionally the effect of *de novo* protein synthesis on coccoid formation was examined by incubation in the presence of 200 μg/ml chloramphenicol (Figure 4.3).

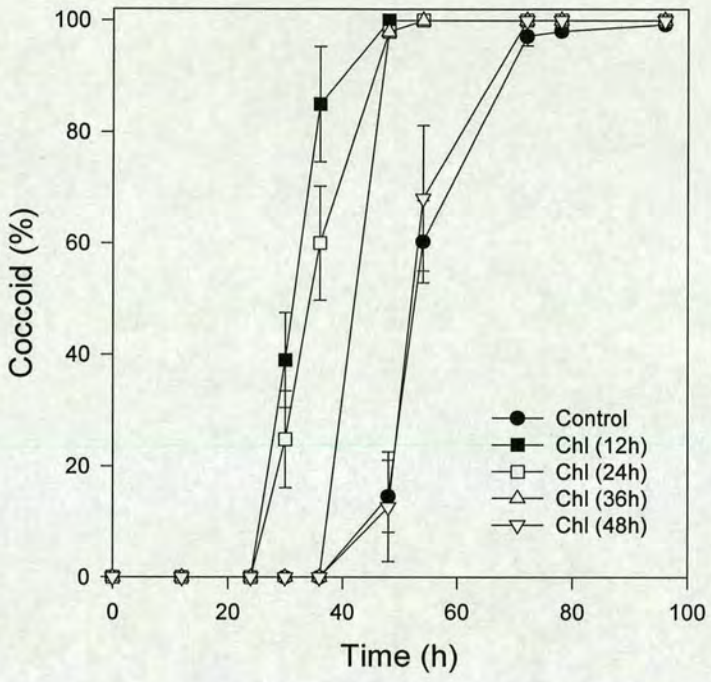


Figure 4.3: Effect of chloramphenicol addition at various times during the growth curve on the rate of coccoid transformation in *Campylobacter jejuni* 81116. Chl = chloramphenicol. Mean of two separate experiments

Chloramphenicol addition prevented cell replication, but not coccoid formation, indicating that *de novo* protein synthesis is not required for this process (Figure 4.3). This confirms reports in the literature (Hazeleger *et al.*, 1995 and Thomas *et al.*, 1999b). Chloramphenicol addition during exponential (12h) and early to mid-stationary phase (24-36h) resulted in quicker entry to the coccoid state compared with the control. During late stationary phase (48h), chloramphenicol addition resulted in procession of coccoid formation at the same rate as in the control. These results imply that the causative factor(s) of coccoid formation are present within the cell as early as exponential phase (12h) and that cessation of cell growth appears to trigger the passive transformation process in a temperature dependent manner (Figure 5.1; Hazeleger *et al.*, 1995).

The effect of formaldehyde (2% v/v) and boiling on coccoid formation at 37°C was monitored (Table 4.2).

Time (h)	0	12	24	36	48	60	72	96	120
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	8.1 ± 3.2	71.8 ± 8.9	97.4 ± 1.6	99.9 ± 0.1	99.9 ± 0.1
Formaldehyde	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Boiling	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 4.2: Effect of formaldehyde treatment and boiling *Campylobacter jejuni* 81116 cells on the rate of coccoid transformation

Formaldehyde addition and boiling of exponential phase cells (12h) prevented morphological transition from the spiral to the coccoid state. This is consistent with the proposal of an unidentified enzyme (possibly cell wall modifying) being responsible for the transition (Amano & Shibata, 1992). Both treatments inactivate most enzymes, indicating that the enzyme is heat-labile. The best candidate for such an enzyme would be an autolysin(s). An autolysin has recently been discovered in *C. upsaliensis* (Santiwatanakul & Kreig, 1999). Furthermore, modification of peptidoglycan was observed during coccoid transition in *H. pylori* cells (Costa *et al.*, 1999).

4.2 Photographic and flow cytometric analysis of growth phase dependent morphological changes

At various points in the growth curve, samples were taken and analysed by photography, in conjunction with DAPI-staining to visualise genomic material (Figures 4.41 to 4.44), and by flow cytometry (Figures 4.51 to 4.53).

During exponential phase (12h) the population consisted of short spiral or comma-shaped cells. The genomic material was dispersed throughout the cell. Many of the cells doubled in length upon entry into early stationary phase (24h), creating a heterogeneous population of elongated cells and short spirals. As evidenced from the

DAPI-stain, the genomic material was dispersed throughout the cell (Figure 4.41). By mid-stationary phase (36h), the cells were up to four times the length of exponential phase spirals. Some coccoid cells and short spirals were present. The coccoid cells possessed genomic material, and many of the elongated spirals were binucleate. The population of coccoid cells increased by late stationary phase (48h). The spiral population was heterogeneous with respect to cell length, ranging from two- to six-times the length of exponential phase cells. Many of the elongated spirals possessed two nucleoids, whilst others contained genomic material dispersed throughout the cell (Figure 4.42).

During the decline phase (54-72h), the number of coccoid cells increased further, forming small clumps of three to four cells. Many coccoid cells possessed genomic material. The cell length of the spiral population was in the range 2-10 fold the length of exponential phase cells, although longer cells (up to 80-times) were occasionally observed. These cells displayed heterogeneity in the numbers of nucleoids possessed, ranging from one dispersed nucleoid to up to six condensed nucleoids (Figure 4.43). As the cells were incubated further (96-120h) the plate counts were maintained with 0.01% of the initial population surviving (Figure 4.1). The majority of the cells were coccoid, existing in clumps of up to twenty cells. Spirals were still present, presumably accounting for the plateable fraction. They were predominantly multinucleate elongated spirals, ranging from four- to eight-times the length of exponential phase cells. However, by 120h some spirals had lost cell wall rigidity, indicated by loss of the spiral shape (Figure 4.44).

Many of the morphological changes observed, correspond with reports in the literature (Griffiths, 1993 and Thomas *et al.*, 1999b). Presently no research has examined the distribution of genomic material within *C. jejuni* cells upon entry into

stationary phase. It is evident that as well as the previously reported morphological heterogeneity of stationary phase cultures, considerable heterogeneity exists in the numbers of nucleoids per cell.

The significance and cause of the formation of multinucleate elongated *C. jejuni* cells is presently unknown. Previously elongated cells have been shown to form upon entry into stationary phase (Griffiths, 1993 and Thomas *et al.*, 1999b) and iron depletion (Field *et al.*, 1986), although this is believed to result from decreased growth rate (Leach *et al.*, 1997). The increase in cell size upon entry into stationary phase is unusual. Most bacteria decrease in size (Kolter *et al.*, 1993). The elongated cells lack septa (Field *et al.*, 1986), however the majority appear to segregate the nucleoid producing multinucleate cells (Figure 4.43 and 4.44). In *E. coli*, mutations in a number of genes involved in cell division give rise to multinucleate filaments lacking septa (de Boer *et al.*, 1990). The cell division proteins of *E. coli* act in an orderly fashion, creating a FtsZ-ring at the septum, before recruitment of other cell division proteins attached to the membrane (FtsQAKLNW). Finally FtsI (PBP-3) creates the peptidoglycan of the septum before EnvA creates the septum membranes, thus splitting the two cells. Other proteins (MinCDE) ensure that cell division occurs at the centre of the cell, not at the poles (Rothfield & Justice, 1997). Studying the genome of *C. jejuni* (Section 9.0) indicates that *C. jejuni* possesses many homologues of cell division proteins present in *E. coli*, however, many are absent (FtsQ, FtsL, and FtsN). As the cell division process of *C. jejuni* is examined at the molecular level, no doubt this phenomenon will begin to be understood, and the reasons for the absence of certain cell division genes made clear. It remains to be discovered whether the phenomenon of elongation is a quirk of laboratory culture, or fulfills a specific purpose.

The importance of elongated cells is unknown, however, they have been observed *in vivo*. Colon-colonising cells in a mouse model were observed to be 3-6 times the length of exponential phase cells (Merrell *et al.*, 1981). This is perhaps due to the slow growth rate of cells in the intestine. However, it is possible, as with stationary phase cells of other bacteria (Huisman *et al.*, 1996), that the elongated spirals are more resistant to environmental stress than exponential phase cells. This will be examined in Section 6.4, with respect to hydrogen peroxide and trisodium phosphate.

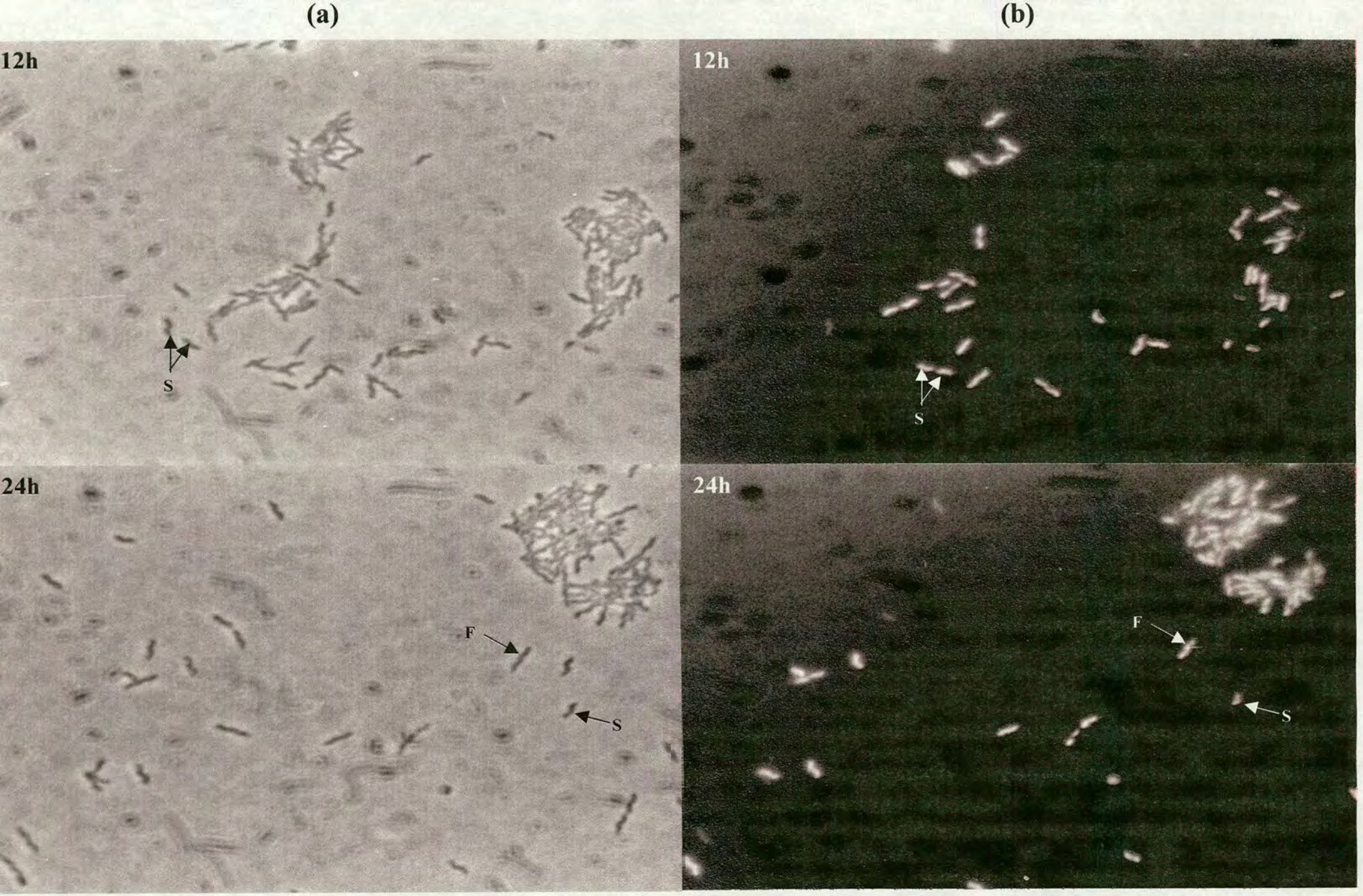


Figure 4.41: Morphology of *Campylobacter jejuni* 81116 cells at exponential phase (12h) and early stationary phase (24h) during growth in Brucella-FBP broth under microaerobic conditions at 37°C (a) phase contrast, and (b) DAPI-stain. S = short spiral; F = filament.

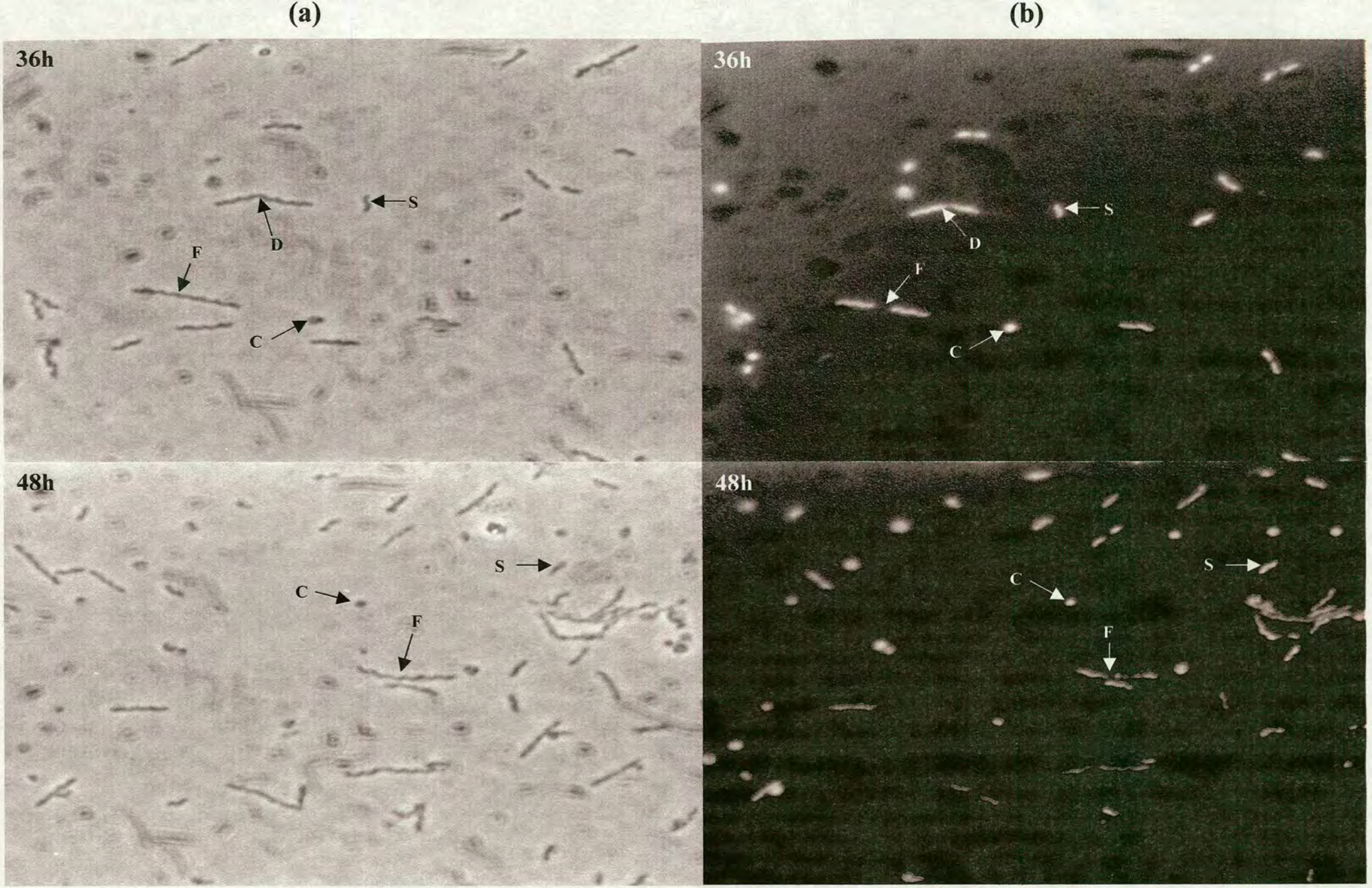


Figure 4.42: Morphology of *Campylobacter jejuni* 81116 cells at mid-stationary phase phase (36h) and late stationary phase (48h) of growth in Brucella-FBP broth under microaerobic conditions at 37°C (a) phase contrast, and (b) DAPI-stain. S = short spiral; F = filament; D = dividing cell; C = coccoid

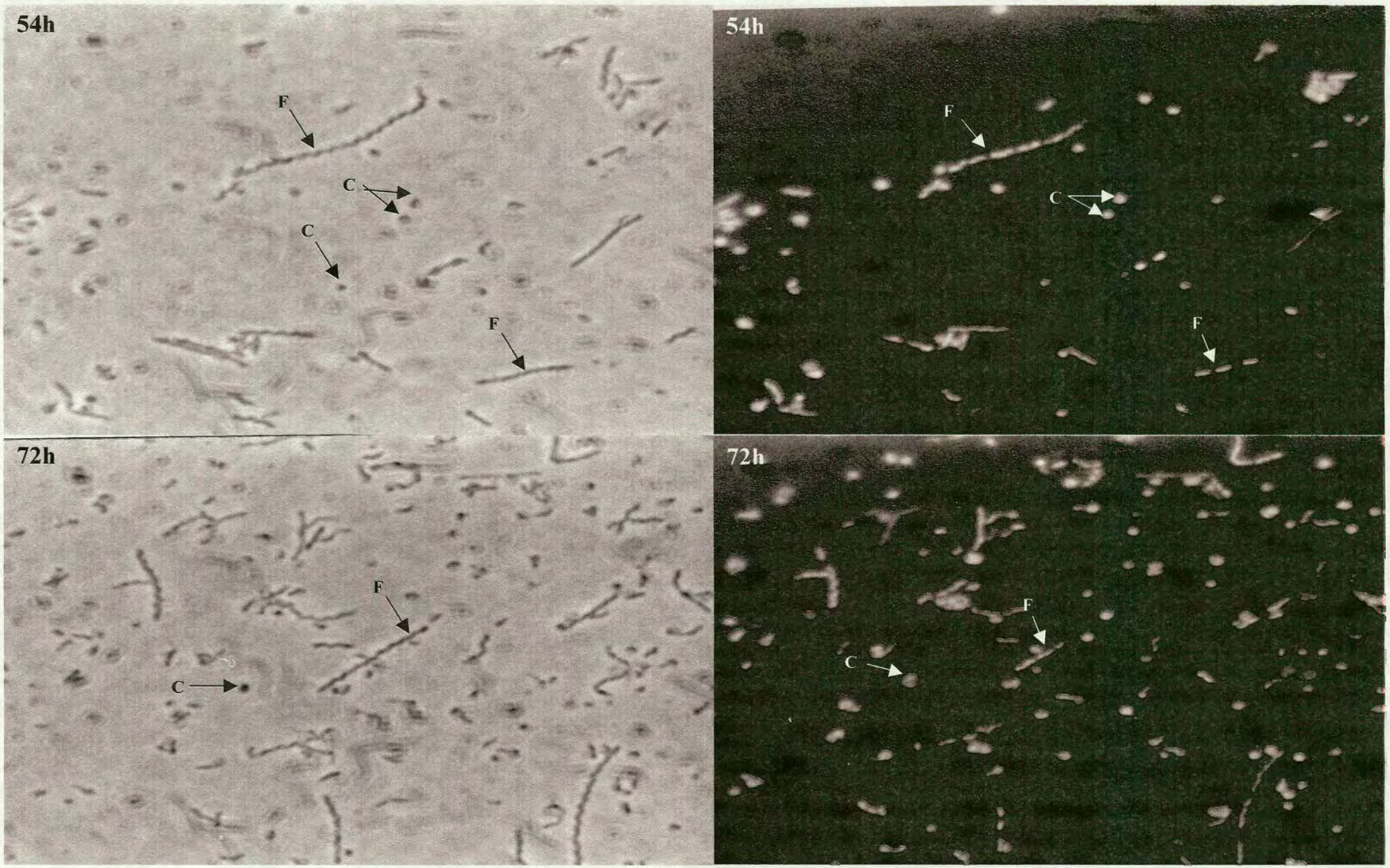


Figure 4.43: Morphology of *Campylobacter jejuni* 81116 cells at early decline phase (54h) and late-decline phase (72h) of growth in Brucella-FBP broth under microaerobic conditions at 37°C (a) phase contrast, and (b) DAPI-stain. F = filament; C = coccoid

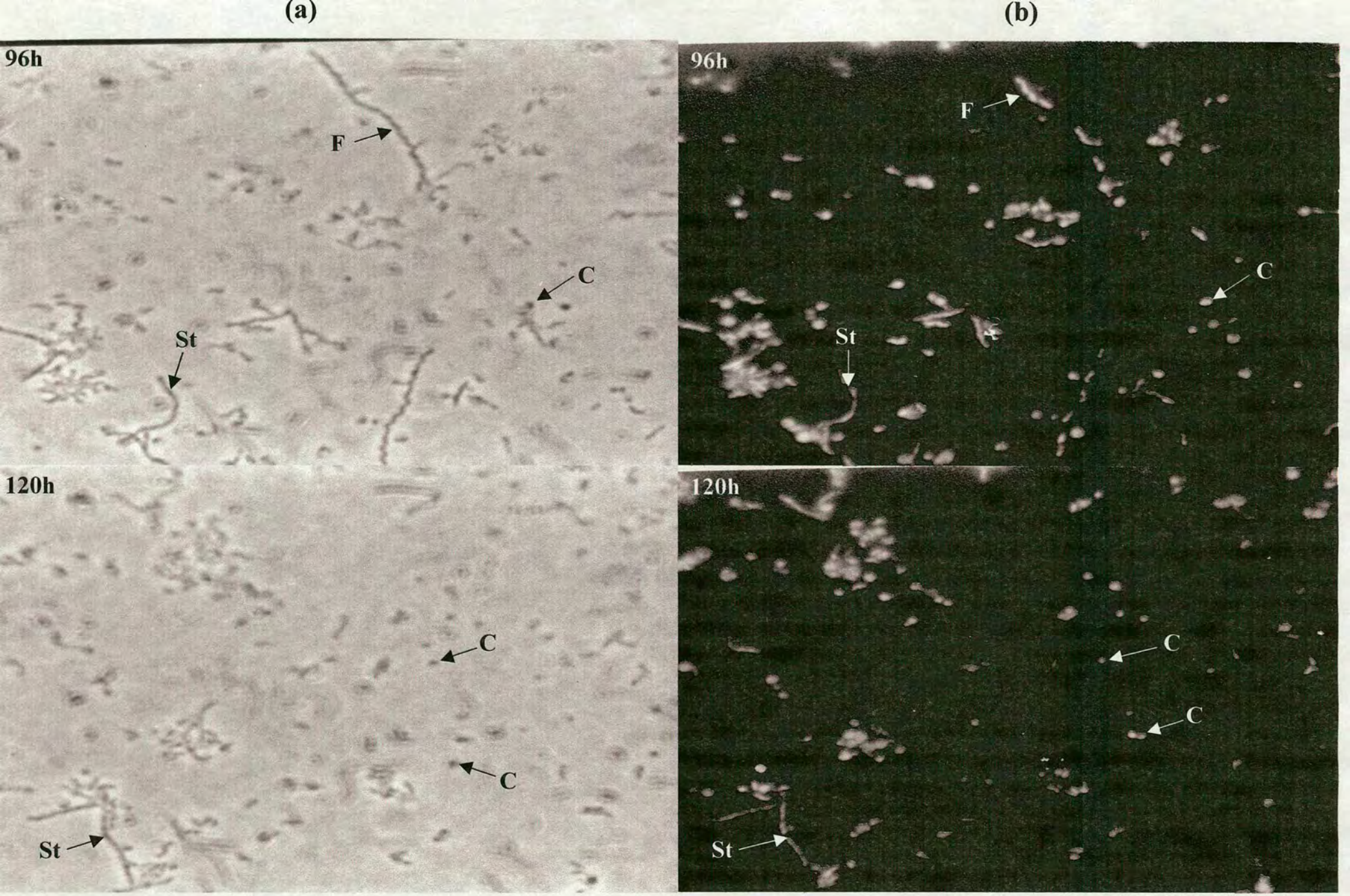


Figure 4.44: Morphology of *Campylobacter jejuni* 81116 cells upon extended incubation in stationary phase (96h and 120h) in Brucella-FBP broth under microaerobic conditions a 37°C (a) phase contrast, and (b) DAPI-stain. F = filament; C = coccoid; St = spiral showing loss of structural integrity

Flow cytometry permits cells to be distinguished on the basis of forward scatter (cell size) and side scatter (cytoplasmic complexity). It was hoped that separate spiral and coccoid cell populations could be detected (Figures 4.51 to 4.53).

During exponential phase (12h), a single peak was observed (Figure 4.51), indicating a homogeneous cell population of short spirals (Figure 4.41). Upon entry into early stationary phase (24h), three populations of cells were evident, designated 1, 2 and 3 (Figure 4.51). In addition to the predominant, short spiral population (1; FS log 5, SS log 0.5), there was a population of cells displaying increased cytoplasmic complexity (2; FS log 5-15, SS log 5-10). Some of the cells had increased in cell length indicated by an increase in forward scatter. Hence these cells are likely to correspond to the elongated spirals, twice the length of exponential phase cells (Figure 4.41). A third, smaller population is present (3; FS log 10-80, SS log 1-5). This population has similar cytoplasmic complexity to population 1, but shows increased cell length. This could indicate a second population of elongated cells, differing in cytoplasmic complexity, or merely be clumps of cells from population 1.

By mid-stationary phase (36h), population 2 had decreased in size, although the length of the cells present in population 2 and 3 increased further, indicated by an increase in forward scatter (Figure 4.51 and Figure 4.42). Upon entry into late stationary phase (48h), population 2 increased dramatically, possibly correlating with an increase in coccoid cells (Figure 4.42). As the coccoid cells are small, and at this stage are not clumping, it is likely that population 2 is a mixture of coccoid cells (2a; FS 5, SS 5) and elongated cells (2b; FS 10-100, SS 10-100).

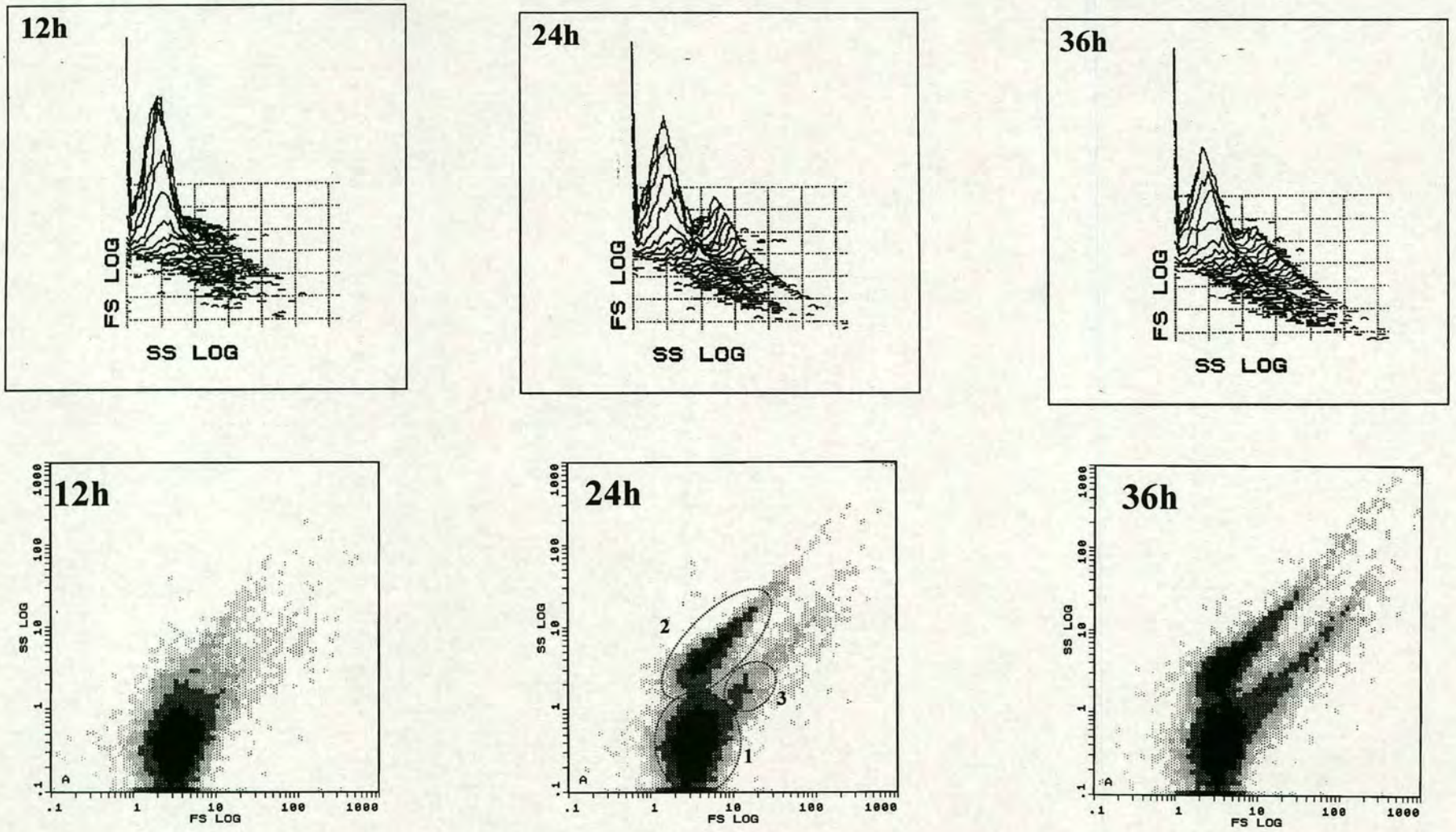


Figure 4.51: Flow cytometric profiles of different growth phases (exponential phase; early stationary phase; mid stationary phase) from a culture of *Campylobacter jejuni* 81116 grown in Brucella-FBP broth under microaerobic conditions at 37°C. FS = forward scatter (cell size); SS = side scatter (cytoplasmic complexity)

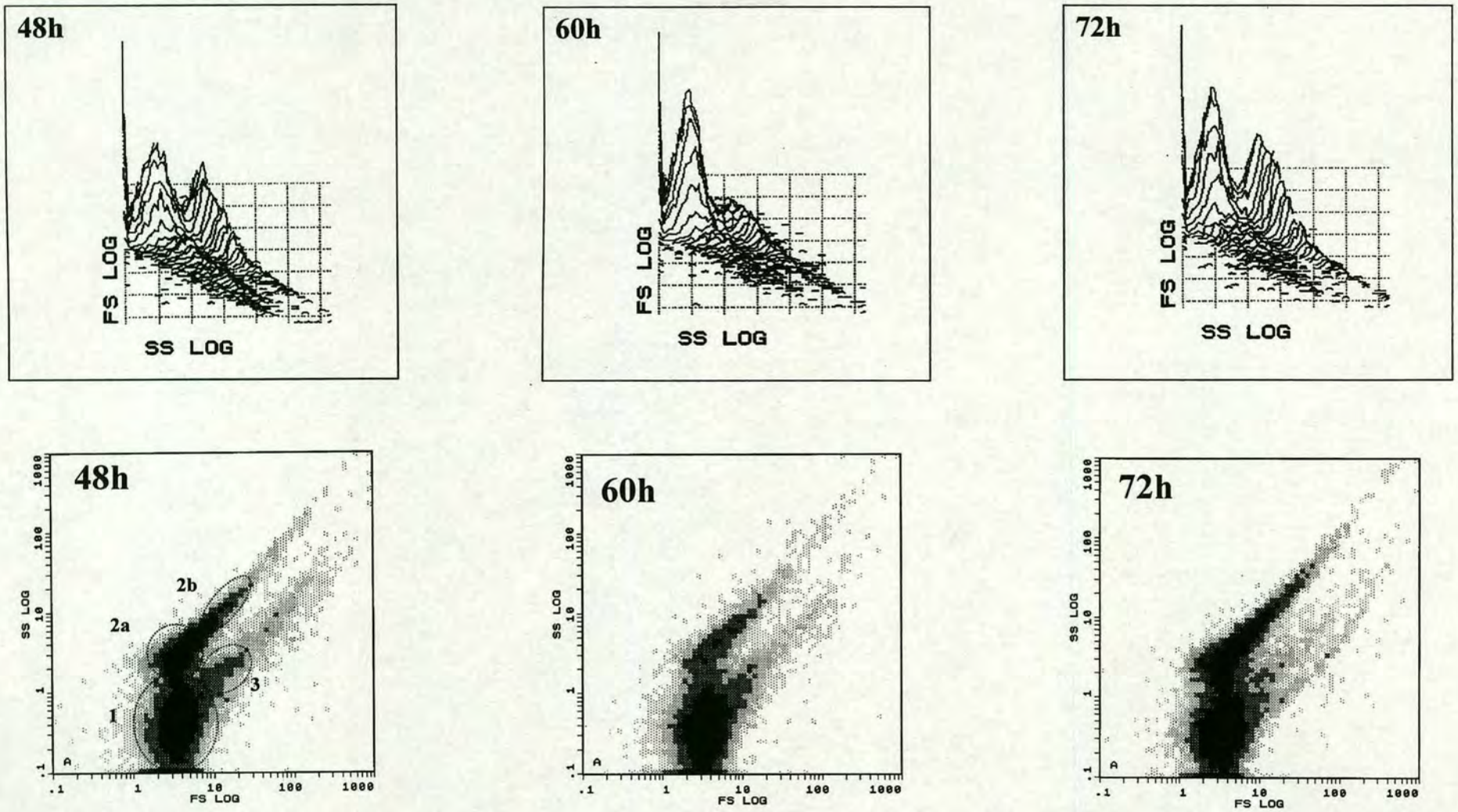


Figure 4.52: Flow cytometric profiles of different growth phases (late stationary phase; early decline phase;late decline phase) from a culture of *Campylobacter jejuni* 81116 grown in Brucella-FBP broth under microaerobic conditions at 37°C. FS = forward scatter (cell size); SS = side scatter (cytoplasmic complexity)

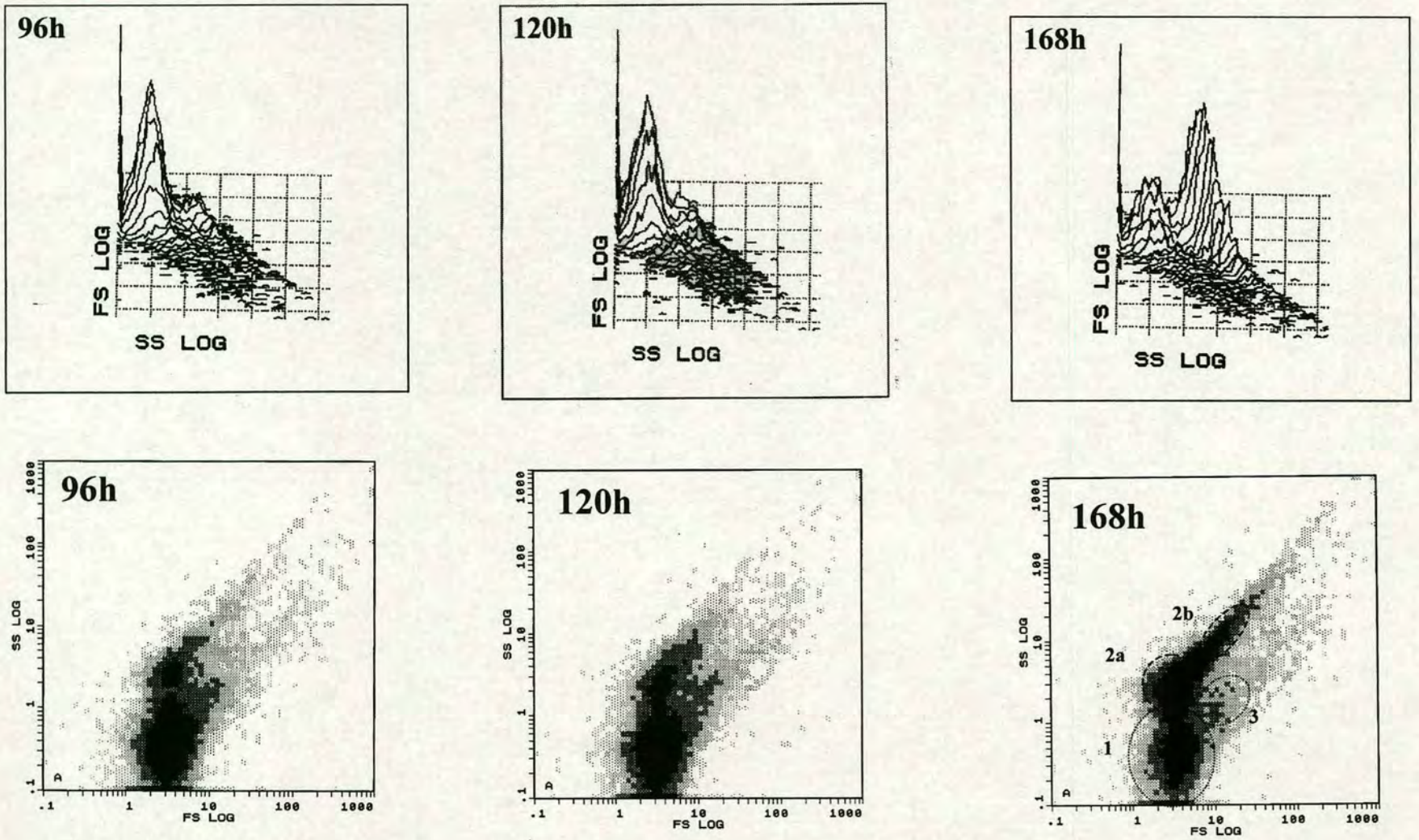


Figure 4.53: Flow cytometric profiles of different growth phases (increased entry into stationary phase) from a culture of *Campylobacter jejuni* 81116 grown in Brucella-FBP broth under microaerobic conditions at 37°C. FS = forward scatter (cell size); SS = side scatter (cytoplasmic complexity)

The cell population becomes increasingly coccoid during the decline phase (60h). However, there was not the expected increase in population 2a (Figure 4.52). There were further increases in population 1, as indicated by increases in the length of the elongated cells (Figure 4.43). By the time the cells had entered the late decline phase (72h), population 3 had almost disappeared, concomitant with increases in populations 1 and 2a (Figure 4.52). The population of cells was 85% coccoid at this stage (Figure 4.1 and 4.43). Clearly 85% of the cells are not present in population 2a, hence two populations of coccoid cells may exist, one with high cytoplasmic complexity (2a), and one with lower cytoplasmic complexity residing within population 1.

Interestingly, by 96h, when the plate counts had increased again by 3-log units (Figure 4.1), there was a large decrease in populations 2a and 2b (Figure 4.53), despite the overall population being predominantly coccoid (Figure 4.1). This indicates that the coccoid cells are physiologically changing from high to low cytoplasmic complexity. It could be assumed that these cells were accounting for the increase in plate counts. However, a population of 0.7% spirals (mainly elongated spirals) exists, equivalent to $\sim 7 \times 10^7$ cells/ml (assuming the total count is $\sim 10^{10}$ cells/ml), and it is probably these cells which are the plateable fraction. Despite this, the possibility of coccoid cells regaining plating ability can not be excluded.

After 120h incubation, populations 2a and 3 increased again, at the expense of population 1 (Figure 4.53), whilst after 168h incubation, population 2a and 2b had become dominant. Microscopically, the populations were predominantly coccoid, and it is proposed that population 2b consists of clumps of coccoid cells and some elongated spirals (Figure 4.44).

The flow cytometric data (Figure 4.51 to 4.53), in conjunction with the morphological (Figure 4.41 to 4.44) and physiological data (Figure 4.1), indicates that stationary phase in *C. jejuni* is a dynamic process, with great heterogeneity at the morphological and physiological levels. The flow cytometric evidence implies that two coccoid populations exist differing in cytoplasmic complexity. The significance of these two populations is unknown, however, the coccoid cells appear to flux between the two states (compare 120h and 168h in Figure 4.53). The primary aim of identifying single populations of coccoid cells was unsuccessful due to the extreme heterogeneity of such cultures. There is much scope for further flow cytometry work. Usage of nucleic acid stains would enable the heterogeneous nature of the various populations to be ascertained with respect to the numbers of nucleoids per cell (Joux *et al.*, 1997b). Various dyes such as PI, CFW and Rh123 could be utilised to obtain information on the various populations (Mason *et al.*, 1995), perhaps enabling easier distinction and separation than cell size and cytoplasmic complexity alone. Recently, a method for sorting cells showing different response to various activity dyes has been developed using a fluorescence activated cells sorter (FACS). The various physiological states were sorted and examined for their maintenance of plating ability (Nebe-Von Caron *et al.*, 1999). Such an experiment would be invaluable in determining whether coccoid cells are dormant cells (Cappelier *et al.*, 1999a) or the morphologic manifestation of death (Kusters *et al.*, 1997).

4.3 Comparison of the mean generation times of *Campylobacter jejuni* and *Campylobacter coli* strains in Brucella-FBP broth at 37°C under microaerobic atmosphere

The mean generation times (μ) of four *C. jejuni* strains and two *C. coli* strains in Brucella-FBP broth are shown in Table 4.3.

Strain	Mean generation time (μ; mins)	Mean growth rate constant (k; h^{-1})
C. jejuni 81116	47.6 ± 2.6	1.26 ± 0.05
C. jejuni NIC176	48.5 ± 2.3	1.24 ± 0.04
C. jejuni NIC45	44.5 ± 2.3	1.35 ± 0.04
C. jejuni 2877	44.0 ± 3.1	1.36 ± 0.07
C. coli 11366	51.0 ± 2.1	1.18 ± 0.03
C. coli N43	49.5 ± 2.7	1.21 ± 0.05

Table 4.3: Growth characteristics of *Campylobacter jejuni* and *Campylobacter coli* strains in Brucella-FBP broth incubated microaerobically at 37°C. Mean of three separate experiments.

The *C. jejuni* strains had lower mean generation times than the *C. coli* strains. These mean generation times are substantially lower than the 90 min observed in a published study (Rollins *et al.*, 1983). However, that study used optical density readings to determine the mean generation times, and as indicated in Section 4.1, they do not correlate with plate counts, due to the large increase in biomass upon entry into stationary phase as the cells increase in cell length (and volume).

4.4 Temperature growth range of *Campylobacter jejuni* 81116

C. jejuni 81116 was grown at various temperature ranging from 4 to 60°C for a period of one week and the growth rates determined (Figure 4.6).

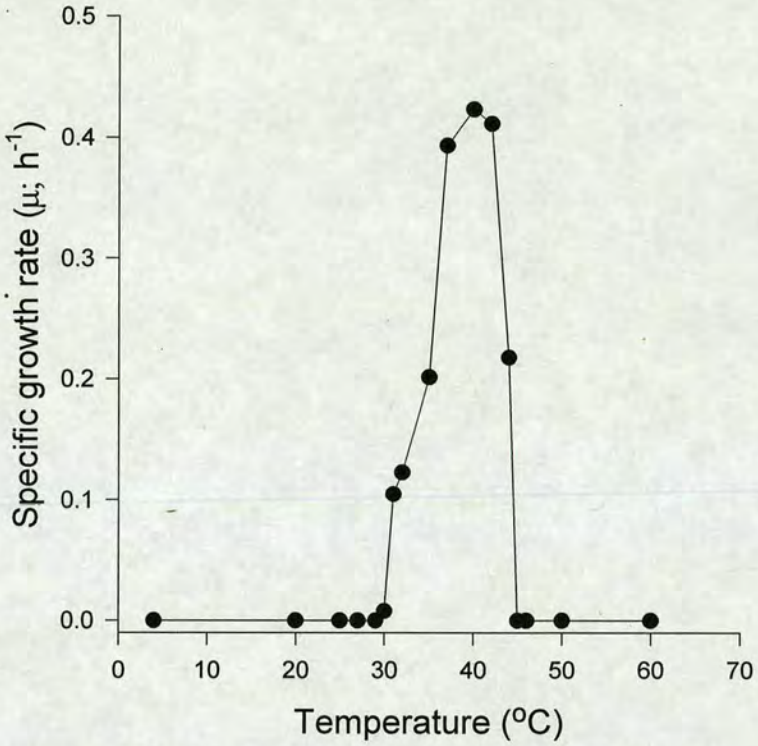


Figure 4.6: Temperature growth range of *Campylobacter jejuni* 81116 in Brucella-FBP broth. Mean of two separate experiments

The temperature growth range for *C. jejuni* 81116 was 31-45°C, with an optimum of 42°C. This compares favourably with published reports (Doyle & Roman, 1981 and Hazeleger *et al.*, 1998). Sharp declines are observed at the maximum and minimum growth range. This phenomenon has been observed previously and attributed to the absence of cold-shock proteins, or structural changes in enzymes regulating growth rate (Hazeleger *et al.*, 1998).

4.5 Growth of *Campylobacter jejuni* 81116 in chemically defined broths

For the purpose of 2D-PAGE studies, cells were required to be grown to mid to late exponential phase in defined media, lacking methionine and cysteine for metabolic labelling experiments. Microaerobic growth of *C. jejuni* 81116 at 37°C was tested in defined media deficient in methionine and cysteine: MEMeagles (Sigma) and ABCD (Pine *et al.*, 1986) broths (Table 2.2). Growth occurred in both Brucella-FBP and ABCD broth. No growth was observed in MEMeagles broth, rather a

decrease in plate counts occurred over 48h. Therefore ABCD broth was chosen as the pulsing medium.

Medium	Incubation time					
	0h	4h	12h	16h	24h	48h
Brucella-FBP	1×10^5	5.7×10^6	1.1×10^9	4.6×10^9	5.4×10^9	4.0×10^8
ABCD	1×10^5	1.4×10^5	8.6×10^7	1.6×10^9	1.9×10^9	5.1×10^8
MEMeagles	1×10^5	5×10^4	6.7×10^3	2.0×10^3	1.0×10^3	6.8×10^2

Table 4.4: Comparison of *Campylobacter jejuni* 81116 growth in complex medium (Brucella-FBP) and two defined media (ABCD and MEMeagles). Mean of two separate experiments.

4.6 Comparison of growth and metabolic labelling in ABCD broth supplemented with various concentrations of Brucella broth

The growth of *C. jejuni* 81116 was compared in ABCD broth (minus methionine and cysteine) supplemented with various concentrations of Brucella broth (Figure 4.7).

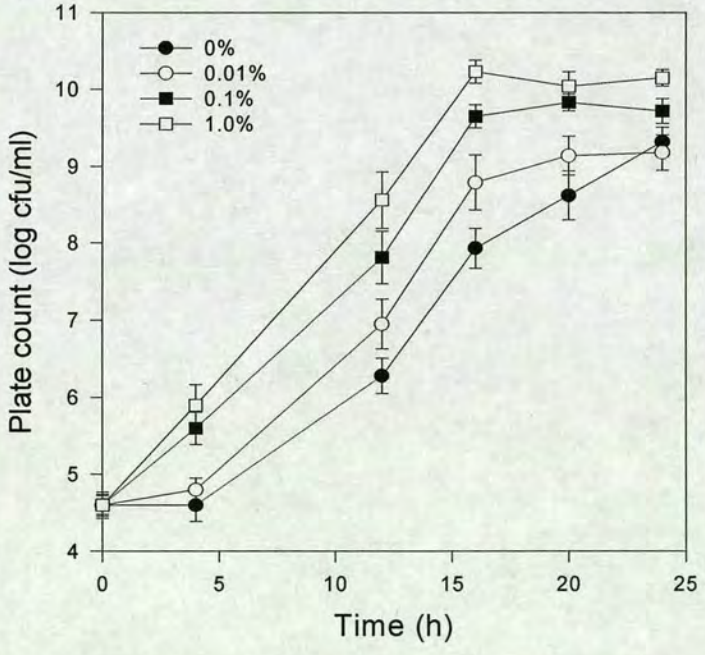


Figure 4.7: Effect of supplementation of ABCD broth (minus methionine and cysteine) with Brucella broth on *Campylobacter jejuni* 81116 growth. Mean of two separate experiments

Addition of 0.01% (v/v) Brucella broth increased the growth rate of the cells (65.1 ± 2.0 min) compared to the control (76.5 ± 3.8 min). Supplementation of ABCD broth with higher concentrations of Brucella broth resulted in loss of the lag phase and increased growth rates compared to the control (Figure 4.7). The labelling ability of cells grown in three supplemented broths was examined (Figure 4.8).

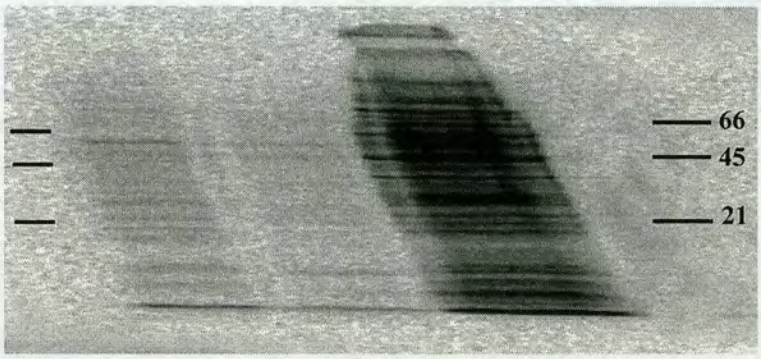


Figure 4.8: Metabolic labelling in ABCD (minus met and cys), ABCD (minus methionine and cysteine; 0.01% [v/v] Brucella broth) and Brucella-FBP broths. Each lane contained 10 μg protein from ~10^8 cells/ml

Metabolic labelling of cells with [^{35}S]-methionine (5 mins at 37°C) occurred in all the media examined, however, growth in ABCD broth with 0.01% Brucella broth gave substantially better labelling. The poor labelling in Brucella broth and ABCD (0.1%) broth could be attributed to competition with excess cold methionine already present within the broths. However, a broth permitting growth and metabolic labelling of *C. jejuni* cells had been constructed.

Growth in ABCD (0.01% Brucella) broth was examined further (Figure 4.9). The cells exhibited a typical growth curve, consisting of a short lag phase (2-4h), presumably where the cells adapt their metabolic pathways to utilise the nutrients present. A 12h exponential phase ensued with a mean generation time of 63 ± 4.3 min, followed by entry into stationary phase (16-44h), concomitant with an increase

in cell length as observed in complex medium (Figure 4.1). Entry into the decline phase ensued after 48h incubation, with a rapid decrease in plate counts (decline rate = 1.7 ± 0.14 h) until the cells were non-plateable. The rapid decrease in plate counts corresponded with the cells losing the ability to exclude CFW, and the formation of coccoid cells (compare Figures 4.9a and 4.9b). The mean generation time for *C. jejuni* 81116 in ABCD broth (63 min) was slower than in Brucella-FBP broth (47.6 min), perhaps as a result of the omission of methionine and cysteine, although some would be carried over in the inoculum, and the added supplement (0.01% Brucella broth). Another possibility is that the complex medium would contain higher concentrations of amino acids such as, serine, which *C. jejuni* uses as a carbon source (Leach *et al.*, 1997), enabling maintenance of a quicker growth rate.

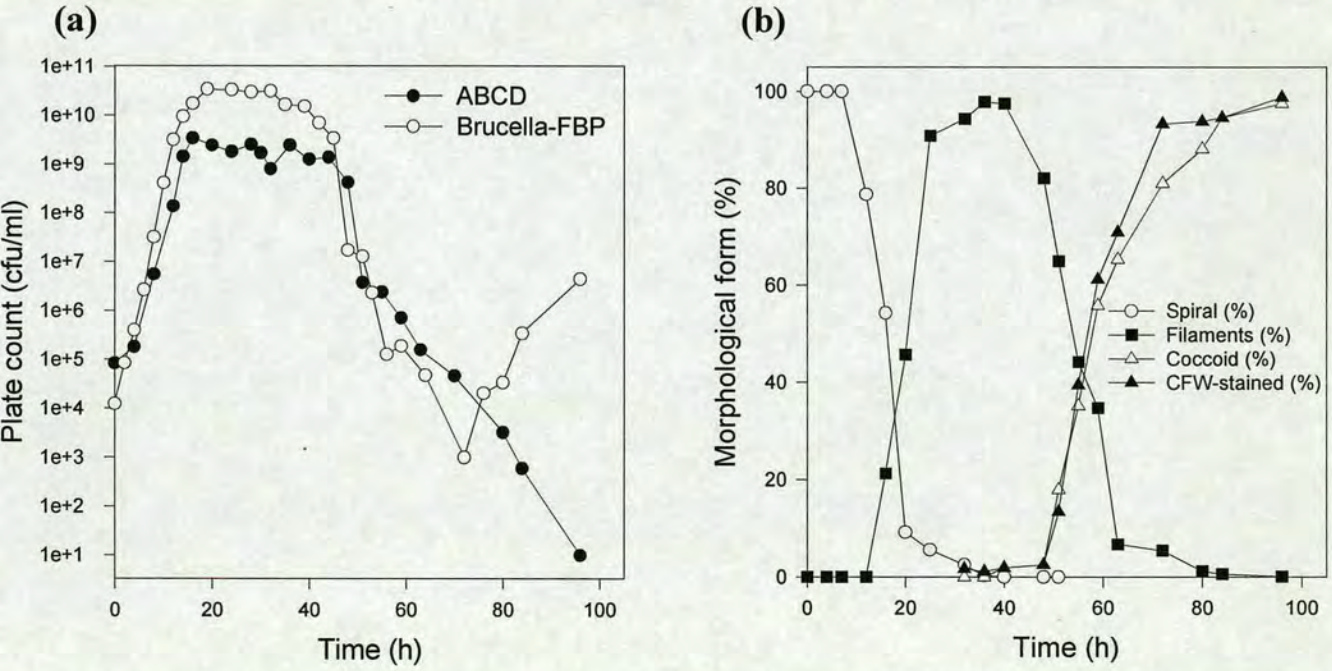


Figure 4.9: Growth characteristics of *Campylobacter jejuni* 81116 in ABCD broth (a) plate counts, and (b) morphology. Mean of two separate experiments

Stationary phase cell densities were higher in Brucella-FBP broth (~10^{10} cfu/ml) compared to ABCD broth (~10^{9} cfu/ml) indicating that nutrients were either absent or present in limiting amounts in ABCD broth. Most likely these were amino

acids, possibly serine, as this has proved to be limiting for *C. jejuni* in this medium (Leach *et al.*, 1997). Sufficient growth was obtained in ABCD broth to permit usage as the labelling medium. Approximately 10^8 cells/ml in exponential phase are required for the labelling experiments for 2D-PAGE analysis of *de novo* protein synthesis. This density was obtained within 16h from an initial inoculum of 10^5 cfu/ml.

4.7 Comparison of the rate of coccoid formation in *Campylobacter jejuni* 81116 incubated in defined (ABCD minus methionine and cysteine) and complex (Brucella-FBP) broths

The growth and rate of coccoid formation was examined in defined (ABCD minus methionine and cysteine) and complex broths (Brucella-FBP) over a period of 50h (Figure 4.10).

In accordance with previous results (Figure 4.9), the growth rate in ABCD broth (71.2 ± 5.8 min) was slower than in Brucella-FBP broth (47.3 ± 2.7 min), and higher cell yields were obtained in Brucella broth. This difference may result from the lower concentration of specific nutrients present in ABCD broth. The lag period observed in ABCD broth could result from the synthesis of specific biosynthetic pathways for vital nutrients. Such nutrients are probably present in complex broths.

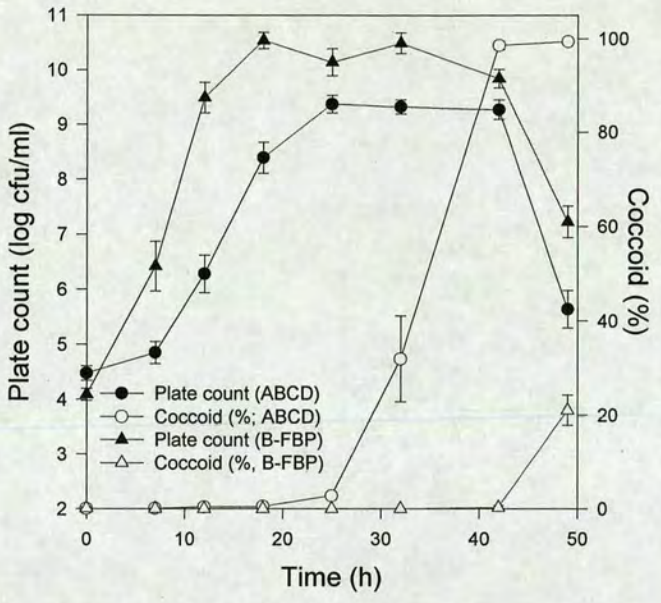


Figure 4.10: Effect of *Campylobacter jejuni* 81116 growth in ABCD and Brucella-FBP broths on the rate of coccoid formation. B-FBP = Brucella-FBP. Mean of two separate experiments

Interestingly, the rate of coccoid formation was more rapid in ABCD broth. The reason for this is unclear, but perhaps results from nutrient limitation, which occurs more rapidly (note differences in growth yields) in ABCD broth. This possibility warrants further investigation. Serine, aspartate, glutamate and proline are the major amino acids utilised by *C. jejuni* (Leach *et al.*, 1997). Whether the absence of these amino acids produces more rapid coccoid transformation compared to the control broth has not been ascertained. Varying the different nutrient concentrations and monitoring coccoid formation could provide insights into a physiological basis for the formation of such cells upon nutrient limitation or entry into stationary phase.

4.8 Summary

C. jejuni was cultured in complex (Brucella-FBP; Figure 4.1) and defined (ABCD; Figure 4.9) broths. Compared to the complex broth, the growth rate was slower in the defined broth and coccoid transformation was more rapid. The temperature growth range of *C. jejuni* 81116 is 31-45°C (Figure 4.6). ABCD broth supplemented with 0.01% (v/v) Brucella broth proved to be an excellent medium for the growth and metabolic labelling of *C. jejuni* (Figures 4.7 and 4.8).

Gross morphological and nucleoid heterogeneity was observed, dependent upon the growth phase (Figure 4.41-4.44). Spiral and coccoid cells could not be easily distinguished by flow cytometry, although distinct physiological populations were detected (Figure 4.51-4.53). The pH of the medium did not affect the rate of coccoid/ filament formation (Figure 4.2). Protein synthesis is not required for coccoid transformation (Figure 4.3).

Section 5

Cold-shock response of *Campylobacter jejuni*

5.0 Results and discussion: Cold-shock response of *Campylobacter jejuni*

5.1 Effect of incubation temperature on plating ability and coccoid transformation

Cells were incubated microaerobically at 4, 20 or 37°C, and their plating ability and rate of coccoid transformation monitored as a function of time (Figure 5.1).

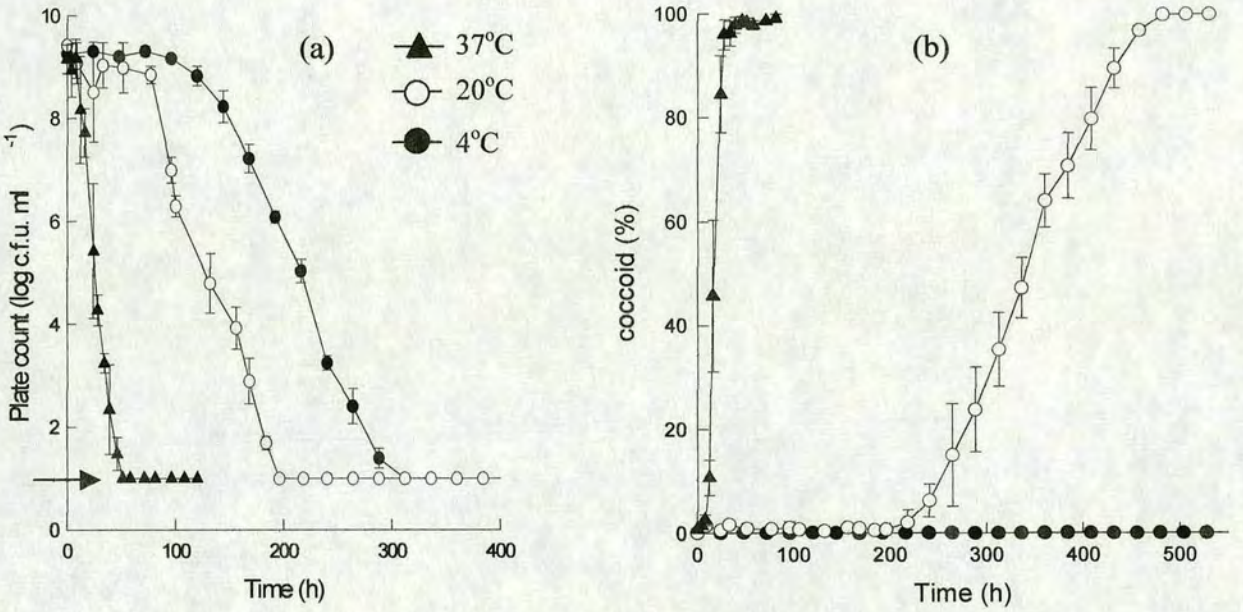


Figure 5.1: Effect of incubation temperature on (a) plating ability and (b) coccoid transformation in *Campylobacter jejuni* 81116. Arrow indicates the limit of detection (10 cfu/ml). Mean of two separate experiments

Decreasing the incubation temperature (37, 20 and 4°C), increases the time required for cells to enter the coccoid state, and prolongs survival in the plateable state (Figure 5.1). These results support previous reports in the literature (Hazeleger *et al.*, 1995). At the lower temperatures (4 and 20°C), entry into the non-plateable state occurs before coccoid transformation, indicating a non-plateable spiral state in the transformation process (Figure 5.2), as recently indicated in the literature

(Federghi *et al.*, 1998 and Lázaro *et al.*, 1999). Indeed, 6 months after starting the experiment, despite the culture being non-plateable, the culture was only 0.1% coccoid at 4°C. Only after 18 months incubation at 4°C had the culture become 70% coccoid (Table 5.1).

Incubation time (months)	6	9	12	15	18
Plate count (cfu/ml)	<10	<10	<10	<10	<10
Coccoid (%)	0.1 ± 0.05	6.8 ± 2.1	21.9 ± 9.5	52.3 ± 10.8	70.2 ± 8.7

Table 5.1: Effect of long-term incubation at 4°C on the rate of coccoid formation of *Campylobacter jejuni* 81116

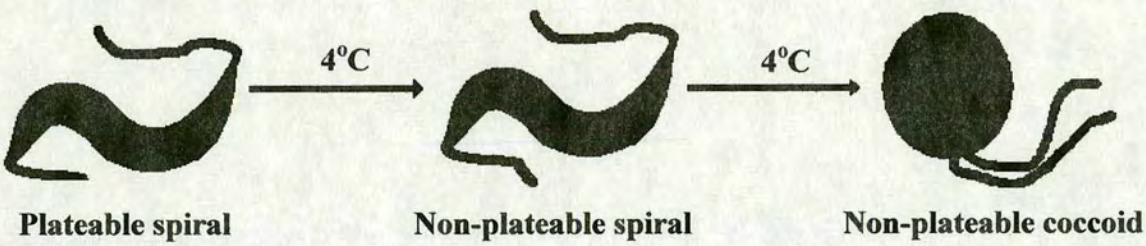


Figure 5.2: Diagrammatic representation of the morphological transition experienced by *Campylobacter jejuni* at low temperature

5.2 Effect of cold-shock at 4°C on the activity of plateable and non-plateable cells

Cells were grown in Brucella-FBP broth to late exponential phase (10^9 cfu/ml) under optimal conditions. Upon cold-shock at 4°C (Figure 5.3), three distinct phases were observed:

1. ***Plateau phase***: lasting approximately 5 days where the plate counts were maintained at their original levels.

2. ***Decline phase***: logarithmic decline to below the detection limit (10 cfu/ml), within 6-8 days with a decline rate of 6.0 ± 0.46h. Together the plateau and decline phases constitute the ***plateable phase***.

3. ***Non-plateable phase***: plate counts remain below the detection limit.

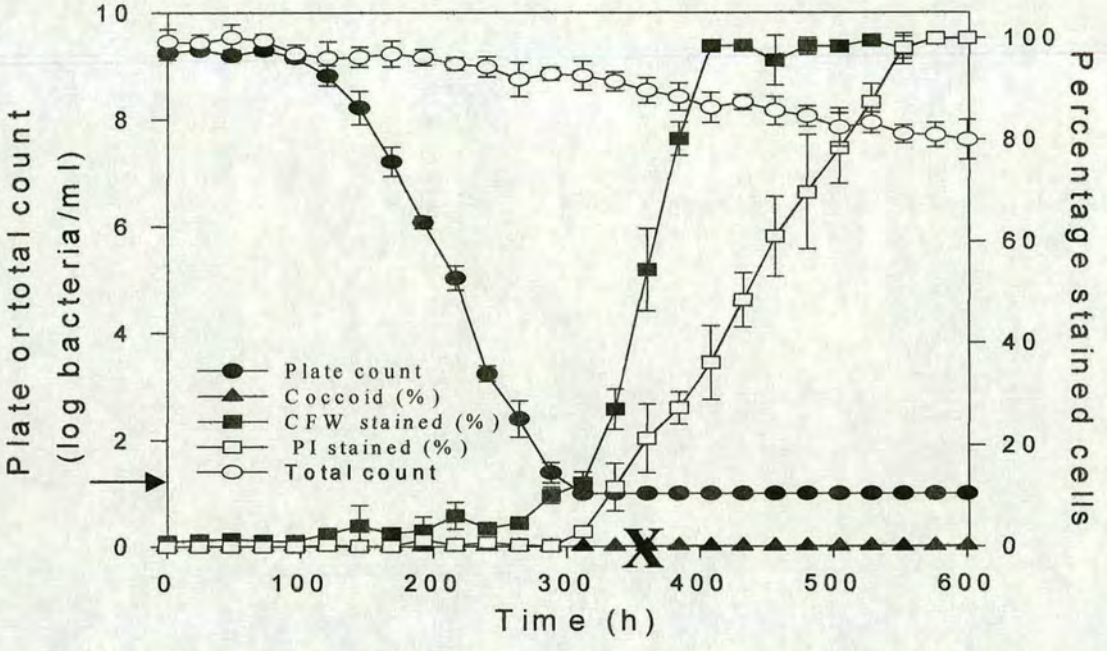


Figure 5.3: Survival of *Campylobacter jejuni* 81116 at refrigeration temperature. Arrow indicates the detection limit (10 cfu/ml). CFW = calcofluor white; PI = propidium iodide. X = point at which resuscitation is attempted in Section 5.3. Mean of three separate experiments.

The total count decreased by ~1 log unit (Figure 5.3), indicating lack of substantial cell lysis, and the formation of a non-plateable fraction. The spiral morphotype (>99.5%) was predominant despite entry into the non-plateable state. CFW stains dead (boiled) cells, but not plateable cells (Section 3.1.1). After the cells entered the non-plateable state, the percentage unable to exclude CFW increased. As the ability of cells to exclude CFW is linked to outer membrane integrity (Section 3.1.3), it appears that non-plateable cells have experienced outer membrane damage.

PI is a nucleic acid stain, but only has access to its binding sites if the cytoplasmic membrane is impaired (Haughland, 1999). Further entry into the non-

plateable state resulted in progressive staining with PI, at a slower rate than the increase in CFW-stained cells (Figure 5.3), indicating progressive structural damage to the cytoplasmic membrane in non-plateable cells. The loss of plating ability was observed not to be the result of changes in the pH of the culture (Table 5.2).

Time (h)	0	50	100	150	200	250	300	350	400	500	600
pH	6.74	6.72	6.75	6.73	6.74	6.73	6.76	6.76	6.75	6.74	6.77

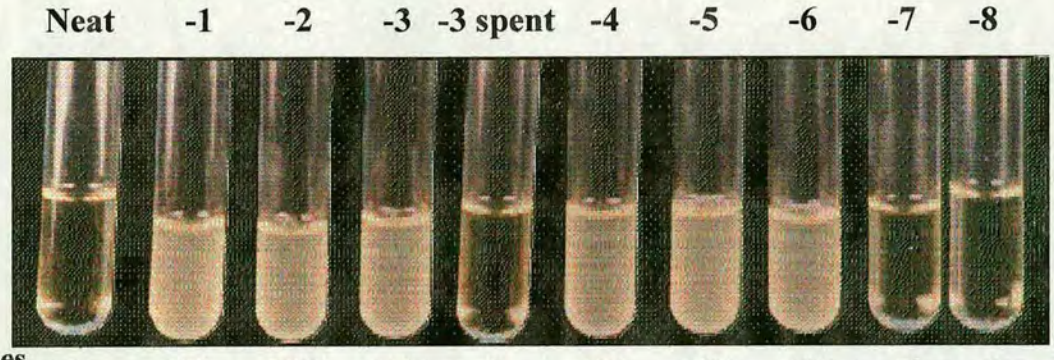
Table 5.2: Changes in culture media pH due to extended incubation of *Campylobacter jejuni* 81116 cells at 4°C

5.3 Effect of resuscitation by temperature upshift and dilution into fresh broth on non-plateable *Campylobacter jejuni* 81116 cells

Once the cells were two days into the non-plateable state, resuscitation was attempted. At this stage, less than 0.2 plateable cells were present in a total population of 1.44×10^9 cells/ml. Therefore the non-plateable population vastly exceeds the plateable one. The majority of the non-plateable cells were metabolically active at this stage, possessing CFW- and PI-excluding ability (Figure 5.3). The non-plateable cells were serially diluted (Table 5.3) into either fresh Brucella-FBP broth or filter-sterilised spent medium, and subjected to a temperature upshift from 4 to 37°C (Figure 5.4).

	Neat	-1	-2	-3	-3 spent	-4	-5	-6	-7	-8
ml	<0.2	<0.02	$<2\times10^{-3}$	$<2\times10^{-4}$	$<2\times10^{-4}$	$<2\times10^{-5}$	$<2\times10^{-6}$	$<2\times10^{-7}$	$<2\times10^{-8}$	$<2\times10^{-9}$
'ml	1.4×10^9	1.4×10^8	1.4×10^7	1.4×10^6	1.4×10^6	1.4×10^5	1.4×10^4	1440	144	14
N	4.9×10^6	4.9×10^5	4.9×10^4	4900	4900	490	49	4.9	0.49	0.049

Table 5.3: Statistical values for plate counts (cfu/ml), total counts (cells/ml) and viable cells estimated by the 'most probable number' (MPN) method (five series of eight ten-fold serial dilutions), present in the dilution tubes used for resuscitation in Figure 5.4.



centage tubes wing growth	0	100	100	100	0	100	100	100	40	0

Figure 5.4: Effect of dilution into fresh or spent Brucella-FBP broth and temperature upshift from 4 to 37°C on non-plateable cells. Percentage tubes showing growth were scored as positive on the basis of turbidity and growth upon plate culture (five tubes per sample)

Growth was observed in all dilutions as high as 10^6-fold, and in 40% of those diluted by 10^7-fold (Figure 5.4). At the time of resuscitation the plate count was less than 0.2 cfu/ml. Upon serial dilution to 10^6-fold this was statistically reduced to $<2 \times 10^{-7}$ cfu/ml (Table 5.3). It is statistically improbable that the observed growth was the result of residual plateable cells. Additionally, the cells had already been two days into the non-plateable state before resuscitation was attempted reducing the risk of residual plateable cells being present. There was a large discrepancy between the numbers of viable cells calculated by the MPN method (Meynell & Meynell, 1965) and the plate counts (<0.2 cfu/ml). The number of tubes showing growth in the 10^{-6}, 10^{-7}, and 10^{-8} dilutions was 5, 2 and 0 respectively. This is equivalent to 4.9×10^6 viable cells/ml (ABNC) present at the time of resuscitation; calculated to be 0.34% of the total population. It appears that the observed growth is due to resuscitation and then division of metabolically active non-plateable cells. Upon dilution it would be expected that the numbers of these active non-plateable cells would decrease to extinction. This was observed to be the case. At the 10^{-7} dilution tube, only 2 out of 5 tubes showed resuscitation, the statistical number of active non-plateable cells

present would be 0.49, however, in reality, the tubes would either contain no cells or one cell (or more). It would be expected that 50% of the tubes would contain at least one cell capable of resuscitation. The actual value obtained was 40% (2/5) which compares favourably. This also indicates that low numbers of active non-plateable cells can resuscitate under the protocol used. The fact that a further ten-fold dilution (10^{-8}) prevented resuscitation indicates the absence of significant dilution errors.

Samples were removed at various intervals from the resuscitation dilution tubes, and the plate counts determined (Figure 5.5). The detection limit on the plates was 10 cfu/ml due to the plating of 100 µl sample. The plate counts in Figure 5.5 are expressed as their statistical value corresponding to Table 5.2, until they are above the detection limit (10 cfu/ml).

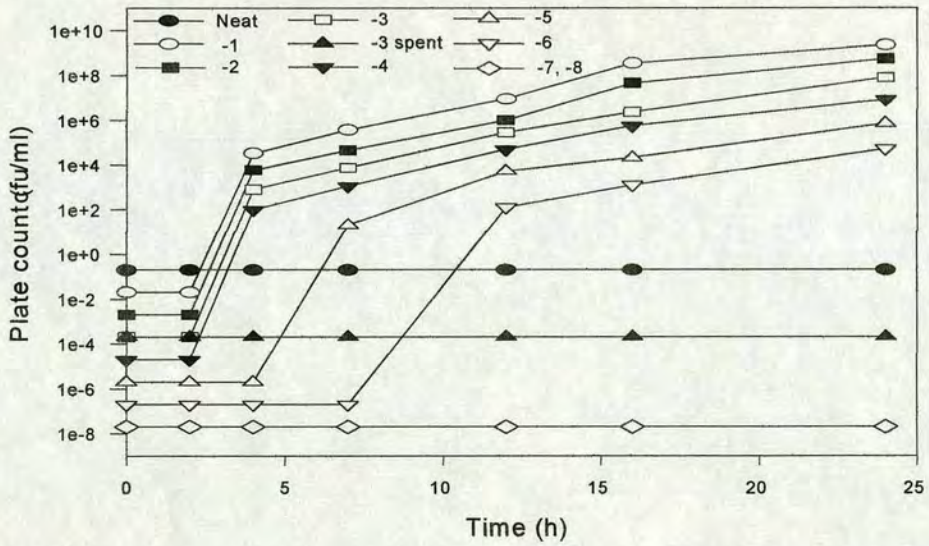


Figure 5.5: Effect of temperature upshift and dilution into fresh broth on cold-shocked, non-plateable *Campylobacter jejuni* 81116 cells as evidenced by plate counts

Non-plateable cells can be resuscitated by a temperature upshift from 4 to 37°C and dilution into fresh medium, after a density-dependent lag period of 4-7h (Figure 5.5). The cells then divide with a growth rate of 48.9 ± 1.8 min. Resuscitation is not observed if the cells are incubated in spent medium (neat). This indicates that either insufficient nutrients are present to permit resuscitation, or an inhibitor is

present preventing resuscitation. The latter was proved correct (Figure 5.8). It is also apparent that at least 288 to 2880 cells are required for resuscitation to occur (Table 5.3 and Figure 5.4 and 5.5), as this density would contain at least one ABNC cell. Analysis of this data provides further evidence that in those tubes showing growth, resuscitation of non-plateable cells had occurred. Assuming the absence of a dilution error, the total number of cells present in the 10^4-fold dilution tube was 2.88×10^5 cells (1.44×10^5 cells/ml), with the possibility of one plateable cell present in the tube determined as 10^{-4}; yet plateable cells were detected within 4h. Assuming one plateable cell was present and the division rate of *C. jejuni* was 47 min, 5.1 divisions would occur within 4h. The number of expected plateable cells would then be ~34, yet the actual number observed was 100, over three times greater than if plateable cells alone had given arise to the observed resuscitation. In conclusion, resuscitation of metabolically active, non-plateable cells (equivalent to ABNC) has been demonstrated at least two days into the non-plateable state. The length of time the cells can be resuscitated, and the effect of different physiological states and gas atmospheres remains to be ascertained.

Was resuscitation due to the growth of low numbers of plateable cells?

A plateable culture (plate count = 6.75×10^9 cfu/ml; total count = 8.1×10^9 cells/ml) was serially diluted by 10^8 to 10^{11}-fold to obtain cultures with between 0 to 100 cfu/ml, and the effect on growth was observed (Figure 5.6) and compared to resuscitated non-plateable cells (Figure 5.5)

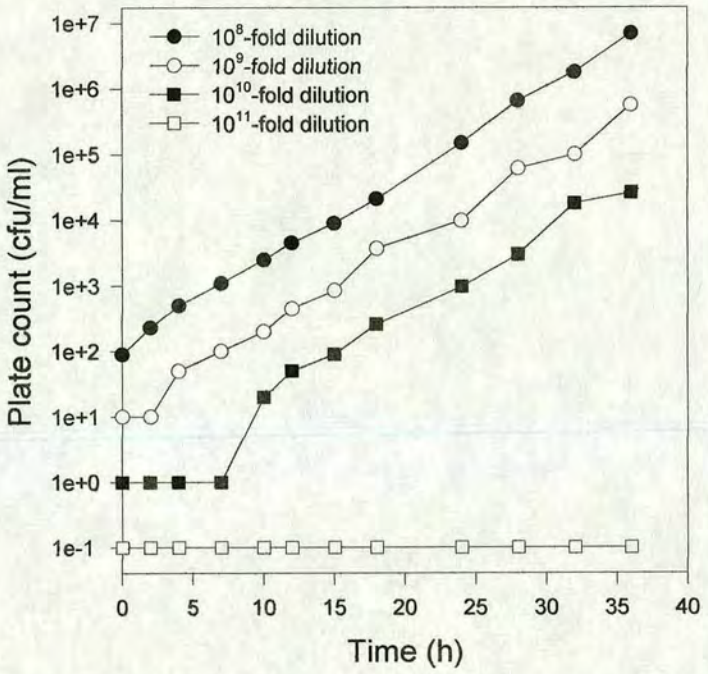


Figure 5.6: Effect of dilution to extinction on the growth of plateable *Campylobacter jejuni* 81116 cells. Limit of detection is 10 cfu/ml. Mean of two separate experiments

The predicted numbers of plateable cells in each tube were 67.5, 6.75, 0.675, and 0.0675 cfu/ml respectively for the 10^8, 10^9, 10^{10}, and 10^{11}-fold dilutions. The actual plate count obtained at 0h for the 10^8-fold dilution was 90 cfu/ml. Dilution errors of at least 10^6-fold would be required to explain resuscitation of non-plateable cells due to the carry over of one residual plateable cell, whilst the measured dilution error was 1.3-fold. No plateable cells were observed in the 10^{11}-fold dilution, containing ~0.0675 cfu/ml. This indicates that dilution of non-plateable cells to statistically below 0.1 cfu/ml would result in tubes possessing no plateable cells. In the previous experiment on resuscitation of non-plateable cells, less than 0.2 cfu/ml was present in the initial culture prior to serial dilution (Section 5.3).

Despite the number of plateable cells being below the limit of detection in the 10^9 and 10^{10}-fold dilutions, it is evident that the plateable cells immediately begin dividing (Figure 5.6). This can be confirmed by extrapolating the plot backwards to time 0h, where the initial plate counts correspond favourably to the predicted value of

6.75 or 0.675 in the case of the 10^9 and 10^{10}-fold dilutions respectively. This is not the case with non-plateable cells (Figure 5.5), where extrapolation of the plot backwards to time 0h, reveals a 2-3 log difference between the predicted number of plateable cells derived from the detection limit (0.2 cfu/ml), and the value obtained from extrapolation. This can be explained by the existence of a population of non-plateable cells that can revert to the plateable state upon subjection to the resuscitation protocol.

Effect of resuscitation in the presence of ampicillin and chloramphenicol

The effect of 200 μg/ml ampicillin on *C. jejuni* 81116 was examined at 37°C in Brucella-FBP medium under microaerobic atmosphere (Figure 5.7).

The concentration of ampicillin used is inhibitory to *C. jejuni* (Figure 5.7), resulting in a bactericidal effect with a D-value of 76.8 ± 4.2 min. Therefore incubation with 200 μg/ml ampicillin for 60 min at 37°C would reduce the population of dividing cells (residual plateable cells) by ~1 log unit. In a non-plateable culture there may be 0 to 9 cfu/ml present if the limit of detection is 10 cfu/ml. Therefore, incubation with 200 μg/ml ampicillin for 60 min would reduce the numbers of plateable cells by a log unit, hence reducing the presence of plateable cells to less than 1 cfu/ml.

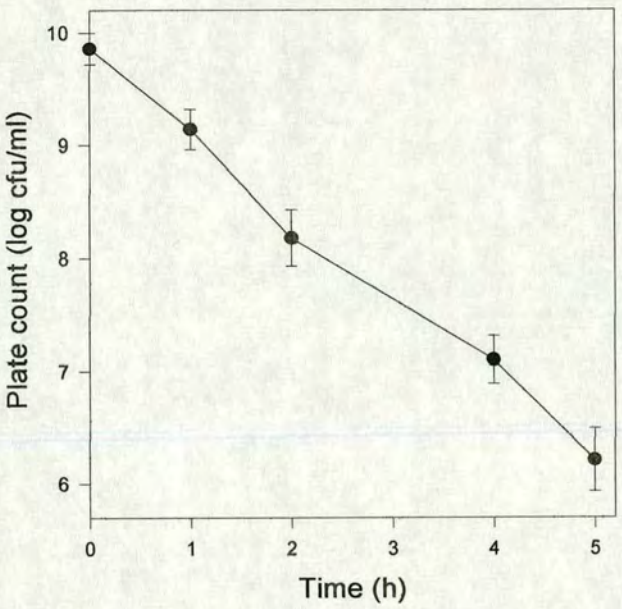


Figure 5.7: Effect of ampicillin (200 μg/ml) on the survival of *Campylobacter jejuni* 81116. Mean of three separate experiments

The effect of ampicillin and chloramphenicol on the resuscitation of non-plateable cells was examined. A population of non-plateable cells was diluted by 10^3-fold and treated with either ampicillin (200 μg/ml) or chloramphenicol (100 μg/ml) for 60 and 30 min respectively at 37°C. For each antibiotic, one set was washed to remove the antibiotic. The cells were then subjected to the resuscitation protocol previously described (Table 5.4).

Antibiotic	Wash	Resuscitation (per 5 tubes)
Control (no antibiotic)	-	5/5
	+	5/5
Ampicillin	-	0/5
(200 μg/ml; 60 min)	+	5/5
Chloramphenicol	-	0/5
(100 μg/ml; 30 min)	+	5/5

Table 5.4: Effect of ampicillin or chloramphenicol treatment on the resuscitation of non-plateable *Campylobacter jejuni* 81116 cells in the presence and absence of washing

Ampicillin kills only those cells that are actively synthesising cell wall (dividing cells). Hence non-dividing (dormant or ABNC) cells would be unaffected (Kaprelyants *et al.*, 1994). Chloramphenicol affects the translation of proteins, hence inhibition of resuscitation in its presence would indicate that *de novo* protein synthesis was necessary for the process.

Resuscitation occurred after treatment with both antibiotics, but only if the cells were washed prior to resuscitation. Therefore, in addition to the cells being diluted by 10^3-fold from an initial value of less than 0.2 cfu/ml, resuscitation after treatment with ampicillin supports the proposal that recovery of non-plateable cells has occurred. Any residual dividing plateable cells present in the resuscitation medium would be reduced by a further log unit (Figure 5.7). The fact that resuscitation only occurred when the cells were washed to remove the ampicillin implied that peptidoglycan synthesis is required for resuscitation.

Incubation with chloramphenicol in the absence of washing prevented resuscitation. This implied that *de novo* protein synthesis was required for resuscitation. Cells were only incubated in the presence of the antibiotic for 60 min at 37°C, hence future work could concentrate on elucidating the kinetics of resuscitation with respect to the synthesis of nucleic acids, proteins and peptidoglycan. These events are most likely to occur within the 4-7h lag period prior to the observed increase in plate counts (Figure 5.5). Protein synthesis and RNA synthesis could be observed by the incorporation of [^{3}H]-methionine and [^{3}H]-uridine as a function of time (Clements & Foster, 1998). The production of recovery specific proteins could be elucidated by incorporation of [^{35}S]-methionine and 2D-PAGE analysis. Chromosome replication during recovery could be analysed by staining the DNA with DAPI and analysis using flow cytometry. Addition of rifampicin inhibits DNA

replication, but allow existing rounds of replication to be completed. It is likely that non-plateable cells possess one chromosome, as they are non-dividing, hence the temporal nature of chromosome replication during recovery can be observed by sampling at intervals during the 4-7h lag period (Clements & Foster, 1998).

5.4 Characterisation of the inhibitor

Plateable and cold-shocked, non-plateable cells were incubated in the presence of fresh and spent medium to examine whether the absence of resuscitation in spent medium was due to insufficient nutrients (Figure 5.8).

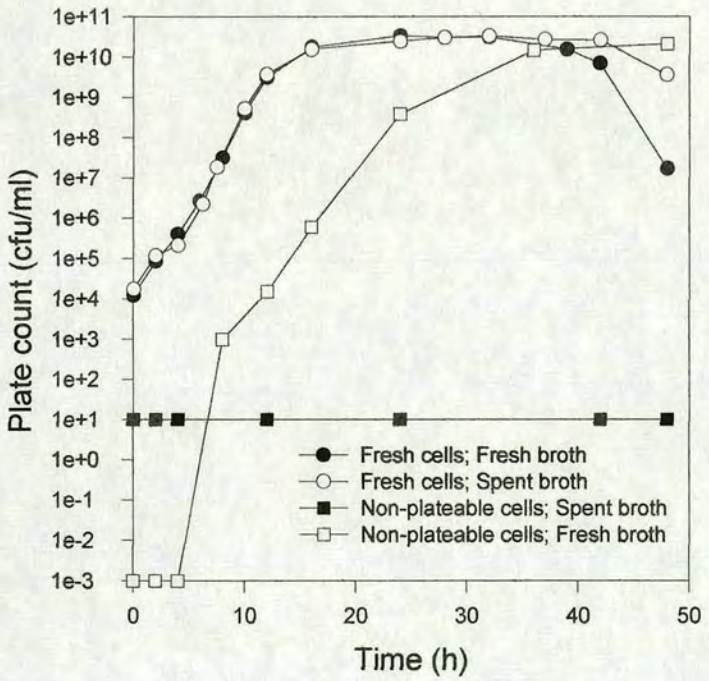


Figure 5.8: Effect of spent and fresh culture medium on the growth of plateable cells and the resuscitation of cold-shocked, non-plateable cells of *Campylobacter jejuni* 81116. Mean of two separate experiments.

As previously observed, non-plateable cells resuscitated upon incubation in fresh medium before multiplying with a doubling rate of 51 ± 2.6 min. Resuscitation was not observed in spent medium. In contrast, plateable cells multiplied in both fresh and spent medium at similar rates of 48.5 ± 2.1 min and 49.2 ± 2.7 min

respectively. This indicates that lack of resuscitation is not due to insufficient nutrients, as it would be expected that the growth rate of plateable cells would be affected. This supports the conclusion that an inhibitory factor is present in the spent medium preventing resuscitation of non-plateable cells. It is also evident that the inhibitory factor does not prevent multiplication of plateable cells.

Non-plateable cells were diluted by 10^3-fold and resuscitated by temperature upshift and dilution into broth with varying ratios of spent and fresh medium (Figure 5.9).

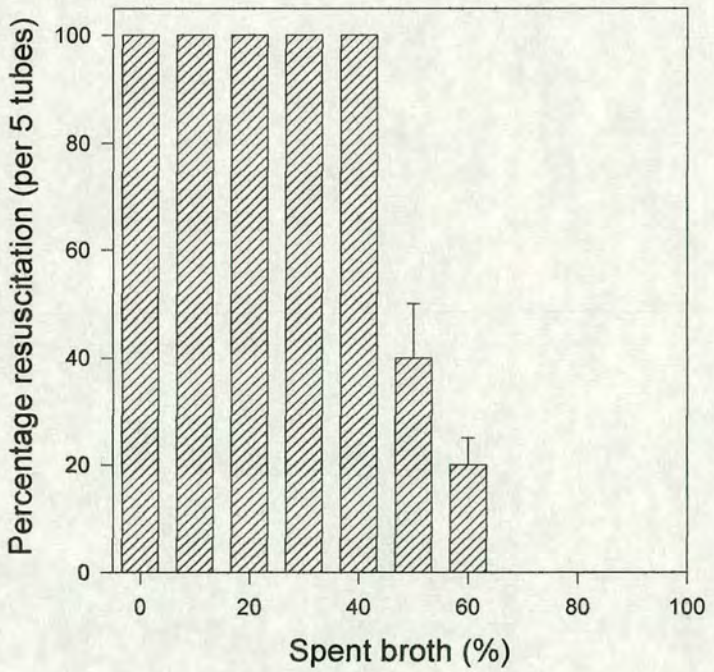


Figure 5.9: Effect of dilution of the inhibitory factor on resuscitation

Dilution of the spent medium by 1.7 to 2.0-fold (50-60% spent medium) resulted in loss of the inhibitory action and subsequent resuscitation. A two-fold dilution factor is extremely small and questions the ecological role of the putative inhibitor, because dilutions much greater would be experienced upon entry into aquatic systems, and thus resuscitation would be expected. It is possible that a combination of temperature upshift, nutrients and dilution of the inhibitor is required

for resuscitation to occur. Entry into aquatic systems would only provide one of these parameters. This is supported by evidence showing that resuscitation only occurs if cells are diluted into fresh broth in conjunction with a temperature upshift to at least 37°C (Tables 5.5 and 5.6).

The ability of cold-shocked (4°C), non-plateable cells diluted by 10^6-fold, to resuscitate upon upshift to various temperatures and dilution into fresh broth was examined (Table 5.5). In addition the effect of nutrient deficient media on resuscitation was examined (Table 5.6).

mperature (°C)	Percentage recovery at each dilution (per 5 tubes)							Plateable cells (+/-)
	Neat	-1	-2	-3	-4	-5	-6	
4	0	0	0	0	0	0	0	-
20	0	0	0	0	0	0	0	-
32	0	0	0	0	0	0	0	-
37	0	100	100	100	100	100	100	+
42	0	100	100	100	100	100	100	+

Table 5.5: Effect of temperature on resuscitation from the non-plateable state

Medium	Percentage recovery at each dilution (per 5 tubes)							Plateable cells (+/-)
	Neat	-1	-2	-3	-4	-5	-6	
S (pH 7.3)	0	0	0	0	0	0	0	-
erile water	0	0	0	0	0	0	0	-
0% B-FBP	0	0	0	0	0	0	0	-
0% B-FBP	0	100	100	100	100	100	100	+
0% B-FBP	0	100	100	100	100	100	100	+
0% B-FBP	0	100	100	100	100	100	100	+
0% B-FBP	0	100	100	100	100	100	100	+

Table 5.6: Effect of nutrient concentration and recovery medium on resuscitation from the non-plateable state upon temperature upshift to 37°C. B-FBP = Brucella-FBP broth

Resuscitation only occurred at temperatures of 37 and 42°C, and in medium containing nutrients greater than 10% (v/v) Brucella broth. Resuscitation at temperatures below 37°C or in nutrient deficient media was not observed even after two weeks incubation, as confirmed by negative growth on plates. It appears that

non-plateable cells do not resuscitate at the environmentally important temperatures of 4 and 20°C that would be found in refrigerators, or upon release into aquatic systems. Similarly resuscitation requires at least a concentration of 30% Brucella broth indicating that resuscitation is unlikely in environmental situations. It should be noted that 30% Brucella broth would still be nutrient rich in comparison to environmental situations. Resuscitation occurred at 37 and 42°C, the body temperature of mammals and avians respectively. It is possible that non-plateable spiral cells could resuscitate within the human or chicken intestine as both nutrients and the relevant resuscitatory temperature would be supplied. This is supported by recent claims that non-culturable spiral cells formed in microcosms held at low temperature could resuscitate within and colonise mice and chickens (Cappelier *et al.*, 1999a).

A number of tests were performed to characterise the inhibitory factor to resuscitation, present within the spent broth. Non-plateable cells were diluted by 10^3-fold and subjected to the resuscitation procedure described in Section 5.3, in the presence of spent medium treated as indicated in Table 5.7.

Test	Resuscitation (per 10 tubes)	Comment
Centricon (excludes >10 kDa)	- (0/10)	Less than 10 kDa
Centricon (excludes >3 kDa)	+ (10/10)	Greater than 3 kDa
Boil (15 min)	+ (10/10)	Heat labile
Acidification (30 min)	+ (10/10)	Acid labile
Proteinase K (100 μg/ml)	+ (9/10)	Proteinaceous
Plus FBP supplement	+ (10/10)	
Minus FBP supplement	+ (10/10)	

Table 5.7: Effect of various treatments on the inhibitory 4°C spent medium

The heat lability, sensitivity to proteinase K and acid, and apparent molecular weight of between 3-10 kDa implies that the inhibitor is proteinaceous. Addition or removal of FBP supplement made no difference to observed resuscitation. The molecular weight of 3-10 kDa would seem to exclude the possibility of a *N*-acyl homoserine lactone, which has been implicated in the resuscitation of dormant *M. luteus* cells (Votyakova *et al.*, 1994), *N*-acyl homoserine lactones are 200-500 Da in molecular weight. Furthermore, screening of *C. jejuni* and *C. coli* cells for N-acyl homoserine lactone-like compounds has been unsuccessful (Swift *et al.*, 1999). However, other signalling molecules isolated from bacteria include, amino acids, cAMP, cyclic dipeptides, butanolides, fatty acid derivatives and short peptides (Rice *et al.*, 1999). From the molecular weight of the inhibitor it would possess in the region of 20 amino acids. However, a 10 amino acid peptide is known to mediate competency in *B. subtilis* via interaction with a histidine kinase in the membrane (Lazazzera & Grossman, 1998). The mechanism and importance of the putative inhibitory factor to resuscitation remains to be elucidated, but certainly warrants further research.

5.5 How wide is the window of resuscitation?

An exponential phase culture was incubated at 4°C under microaerobic atmosphere until it entered the non-plateable state. Non-plateable cells were diluted in the range of ten to 10^8-fold and resuscitated between one and five days after entry into the non-plateable state (Figure 5.10).

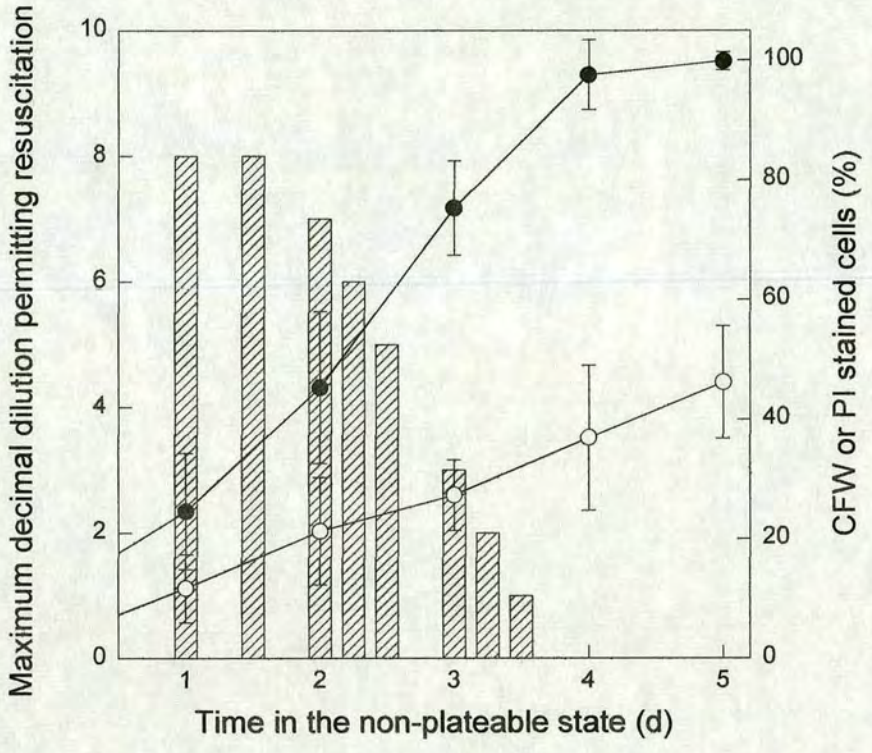


Figure 5.10: Effect of length of time in the non-plateable state on resuscitation. Closed circles = CFW-stained cells. Open circles = PI-stained cells.

Resuscitation occurred up to 3.5 days into the non-plateable state but at varying maximal dilutions, indicating entry into a non-plateable state from which the cells can not be resuscitated by the protocol used. This occurred four days after entry into the non-plateable state, correlating with 98.4 ± 0.7% of the population losing the ability to exclude CFW, indicating outer membrane damage.

Therefore there are two phases of non-culturability, as observed in *V. cholerae* (Wai *et al.*, 1996) and *S. enteritidis* (Roszak *et al.*, 1984). First the cells become non-plateable but can be resuscitated by the protocol used, then upon further incubation at 4°C they enter a state from which they can not be resuscitated. Throughout these experiments the cells were spiral in morphology. Though the

window of resuscitation is small, it does not mean that the cells are dead, as it may merely indicate the limitation of the resuscitation protocol employed.

The transformation model at low temperature (Figure 5.2) can be developed further. The non-plateable spiral state can be divided to distinguish between those, which can be resuscitated, and on their ability to exclude CFW and PI (Figure 5.11).

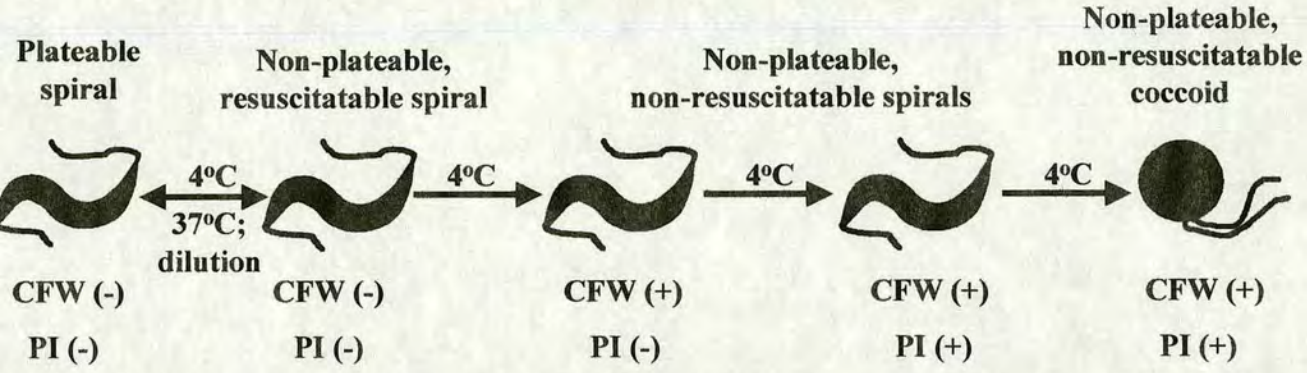


Figure 5.11: Diagrammatic representation of the morphological changes occurring upon low temperature incubation in *Campylobacter jejuni*

5.6 Use of selective and non-selective media for the detection of sublethally injured *Campylobacter jejuni* cells during cold-shock

Upon stress, cells may lose plating ability in the presence of selective agents such as NaCl, antibiotics or bile salts, indicating sublethal injury (Ray, 1984). Cold-shock by either maintenance at low temperature or freezing has been shown to result in sublethal injury in a number of bacteria including *Listeria monocytogenes* (Dykes & Withers, 1999) and *Lactobacillus bulgaricus* (Castro *et al.*, 1997). Cells were incubated at 4°C under microaerobic conditions and the effect of non-selective and selective media on the recovery of the plateable fraction was compared (Figure 5.12). The two selective media used consisted of 1% (w/v) deoxycholate (Humphrey &

Cruickshank, 1985) or an antibiotic cocktail (vancomycin, trimethoprim and polymyxin B) added to Brucella-FBP broth.

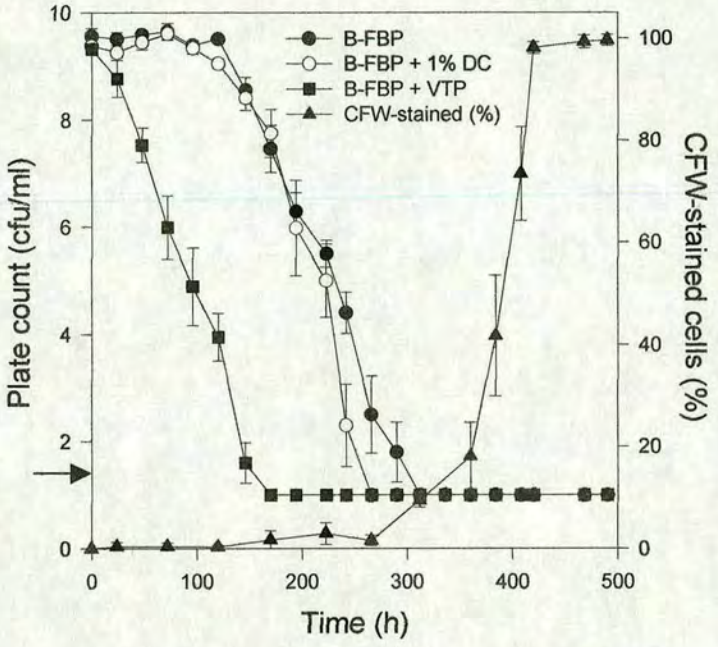


Figure 5.12: Use of selective and non-selective media to determine the effect of cell injury during incubation at 4°C. B-FBP = Brucella-FBP agar, DC = deoxycholate, VTP = vancomycin, trimethoprim and polymyxin B (Skirrow's agar). Arrow indicates the detection limit (10 cfu/ml). Mean of two separate experiments.

The survival curves of the non-selective media and media containing 1% deoxycholate correlated until mid-decline phase (223h; 9.3 days), when the deoxycholate plate counts decreased markedly, becoming non-plateable two days before the non-selective plates. However, plating ability on the antibiotic containing plates decreased immediately, with the culture becoming non-plateable after 7 days, over 6 days before the cells became non-plateable on non-selective media (Figure 5.12). This indicates that a large percentage of the cold-shocked cells are sublethally injured, particularly with respect to their ability to tolerate antibiotics. It is known that cold-shock increases the sensitivity of *C. jejuni* to the antibiotics used in this study (Ray & Johnsen, 1984). Furthermore it is noted that the sublethally injured

cells possess the ability to exclude CFW. This ability is only lost once the cells lose the ability to be cultured on non-selective plates.

Discrepancies between selective and non-selective plates are traditionally believed to indicate sublethal injury (Kell *et al.*, 1998). It is apparent that cold-shocked *C. jejuni* cells are becoming progressively more injured as exposure time increases (Figures 5.3 and 5.12), indicating a continuum of successive injuries, possibly eventually resulting in cell morbidity. The injury manifested by loss of antibiotic tolerance occurs before outer and cytoplasmic membrane damage. Media containing antibiotics are used to isolate Campylobacters from food samples. If the food had been previously chilled, it is evident that the viable population would be underestimated, as both sublethally injured and recoverable non-plateable forms would not be detected.

It appears that the primary site of damage is the membrane. Similar results were observed in freeze-dried *L. bulgaricus* cells (Castro, *et al.*, 1997). In this study, membrane damage was monitored by leakage of ATP, RNA, ATPase activity and changes in the Na^+/K^+ ratios. Similar studies could be performed in *C. jejuni*, however, it is already known that coccoid cells leak ATP and RNA (Beumer *et al.*, 1992 and Moran & Upton, 1986). Additionally dyes indicating loss of membrane potential, such as oxonol could be used. In *L. bulgaricus*, addition of cryoprotectants (skimmed milk, glycerol, trehalose) resulted in increased survival after freezing. Whether the addition of such compounds would aid survival of *C. jejuni* during cold-shock is worthy of research, perhaps providing insight into how *C. jejuni* survives for so long in the absence of protein synthesis at low temperature.

5.7 Effect of cold-shock at 4°C on metabolic staining

Maintenance of functions such as membrane integrity and enzyme activity are possible indicators that non-plateable cells are alive (Roszak & Colwell, 1987a). The same cells used in Section 5.2 were examined for their ability to stain with metabolic dyes (CFW, PI, CFDA and INT) upon incubation at 4°C (Figure 5.13).

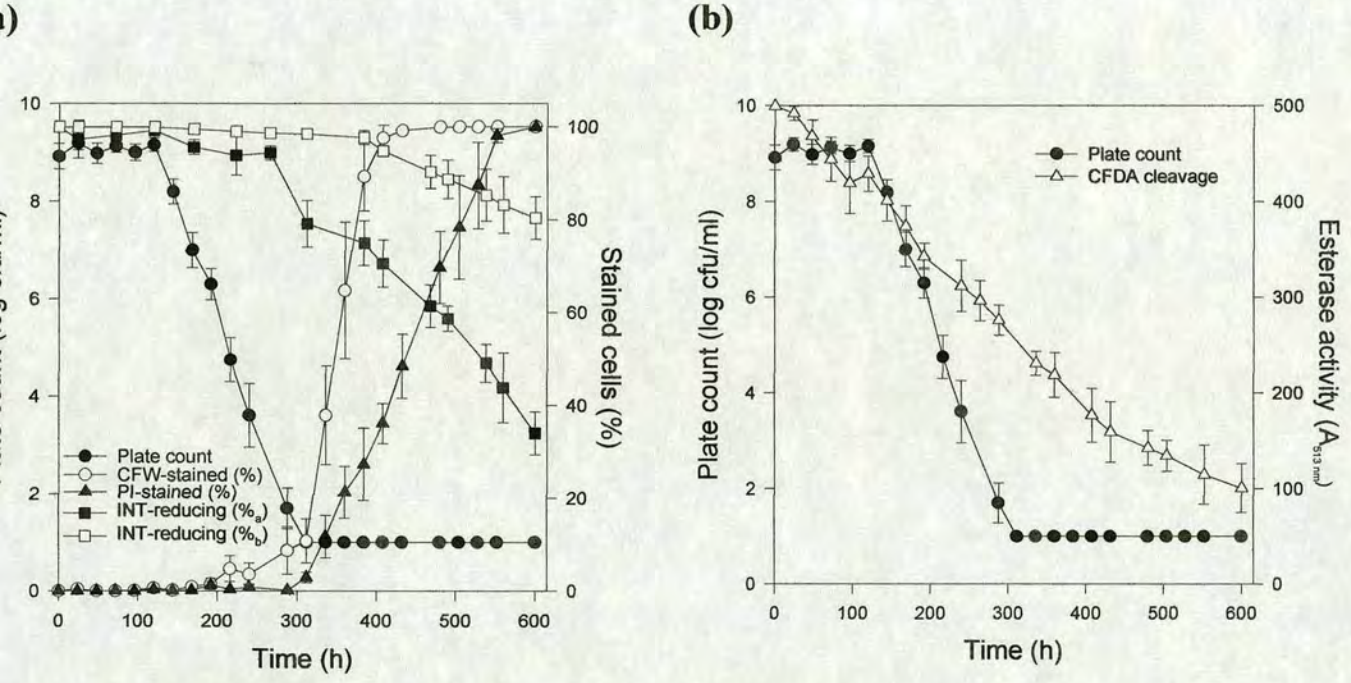


Figure 5.13: Effect of cold-shock on the survival of *Campylobacter jejuni* 81116 as indicated by metabolic stains. (a) CFW, PI and INT, and (b) CFDA. INT%$_a$ = minus succinate; INT%$_b$ = plus succinate. Mean of two separate experiments

The plate counts fell below the limit of detection after ~300h, resulting in the previously observed increase in CFW-stained cells, indicating outer membrane damage. This was followed by an increase in cytoplasmic membrane damage, indicated by increased PI-staining (Figure 5.13a). The percentage of cells reducing INT in the absence of succinate remained >90% for 266h, whilst the cells were still plateable. The percentage of INT-reducing cells then decreased to 34% by 600h. In contrast, addition of succinate stimulated INT-reduction (>80%) in these cells

(Figure 5.13a). Immediately upon cold-shock there is a progressive reduction in esterase activity (Figure 5.13b). Whether this is a result of reduced enzyme activity at 4°C or reduced gene expression is unknown. Esterase activity occurs up to 300h into the non-plateable state (Figure 5.13b).

Cold-shocked, non-plateable cells are not static. They possess the ability to reduce INT in the presence of succinate (an indication of active respiratory dehydrogenases), and esterase activity. This is despite considerable membrane damage that has occurred by 300h into the non-plateable state. Temperature upshift and dilution into fresh broth can not resuscitate such cells. Whether these cells can repair such damage, and whether irreparable damage to the genome has occurred is unknown. It has been shown that prolonged cold-shock of *V. vulnificus* cells resulted in two phases of non-culturability. Initially culturability is lost with maintenance of cellular integrity and intact RNA and DNA (possibly viable), followed by gradual degradation of nucleic acids (Weichart *et al.*, 1997).

The evidence presented at low temperature suggests that as the length of incubation increases, *C. jejuni* cells become increasingly injured, whilst maintaining various enzyme activities. Resuscitation (by temperature upshift and dilution) was not possible once the cells had experienced outer membrane damage. This appears to suggest that the cells are in a process of dying, and that resuscitation is due to the ability of the cells to repair the outer membrane. However, it is feasible that factors other than temperature upshift and dilution are required for resuscitation of such injured cells, and these may be provided by alternative resuscitation protocols such as animal or egg passage (Cappelier *et al.*, 1999a,b). The concept of a time-dependent succession of physiological states has been indicated in starved (Joux *et al.*, 1997b)

and irradiated (Caro *et al.*, 1999) *S. typhimurium* cells, and starved cells of *Deleya aquamarina* (Joux *et al.*, 1997a).

It is evident that whilst much attention has been focused on the difficulties in proving that ABNC cells can resuscitate (and are therefore alive), it is as equally difficult to prove that they are dead (in the absence of lysis). In the future, a suite of activity measurements and resuscitation protocols will need to be utilised from various successful studies in order to elucidate whether ABNC cells (of a particular species) are alive or dead. Candidates for activity measurements should centre on non-enzymatic assays such as the DVC (Joux *et al.*, 1997b), Rh123/ oxonol (membrane potential; Mason *et al.*, 1995), CFW (outer membrane damage), PI (cytoplasmic membrane damage; López-Amorós *et al.*, 1995) and usage of nucleic acid stains in conjunction with flow cytometry to assess genomic damage (Weichart *et al.*, 1997). Possession of an enzyme does not necessarily mean that the cell is 'alive'. Scenarios may arise where the cells are dead, yet structurally intact and possessing enzyme activity due to the intrinsic stability of the enzyme. Resuscitation protocols should prove that no residual plateable cells could result in the observed resuscitation. This is best achieved by allowing the cells to enter the non-plateable state before subjecting them to substantial dilution. A series of *in vitro* and *in vivo* protocols should be used, specific for both the organism and stresses that induced the non-plateable state. Simple reversal of the stress is an obvious starting point, and dilution of the cells dilutes any inhibitors that may be present. Plateable cells may provide factors conducive to resuscitation (Ravel *et al.*, 1995 and Votyakova *et al.*, 1994), therefore resuscitation should be compared in the presence and absence of a low number of plateable cells. One study used heating for 1 min to recover non-plateable *V. cholerae* cells (Wai *et al.*, 1996). Factors such as low pH, and increased

nutrients would be experienced by food-borne pathogens being ingested by animals from an infested water supply. Perhaps similar *in vitro* treatments would permit resuscitation of non-plateable cells. Animal passage has often been cited as resuscitating non-plateable cells (Oliver & Bockian, 1995, Colwell *et al.*, 1996 and Cappelier *et al.*, 1999a), however, care must be taken to dilute the non-plateable culture to prevent inoculating residual plateable cells. Egg passage (Cappelier *et al.*, 1999b) and protozoan ingestion (Steinert *et al.*, 1997) have also indicated recovery of previously non-plateable cells. Finally it should be noted that ABNC cells may not be 'dormant' but the result of a series of progressive injuries, hence the timing of resuscitation may be vital to recover the cells before they enter a truly moribund state. From the perspective of human health it is necessary to assess the ability of non-plateable cells to resuscitate at frequent (every 2-4 days) intervals rather than weeks or months.

5.8 Effect of cell density on survival in the plateable state at 4°C

The rate of entry into the non-plateable state of cells grown to different densities in Brucella-FBP broth upon subjection to cold-shock at 4°C was investigated (Figure 5.14).

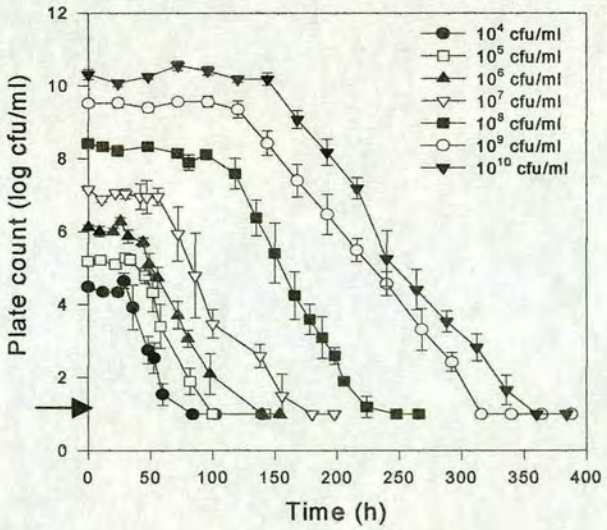


Figure 5.14: Effect of cell density on the survival of *Campylobacter jejuni* 81116. Arrow indicates the detection limit (10 cfu/ml). Mean of two separate experiments.

As the cell density increases, survival in the plateable state increases. Survival is primarily manifested by an increase in the length of the plateau phase although an increase in the decline rate contributes (Table 5.8).

Cell density (cfu/ml)	Plateau phase (hours)	Decline phase (hours)	Decline rate (hours)
10^4	29 ± 4.6	55 ± 6.7	5.0 ± 0.42
10^5	34 ± 3.2	66 ± 4.9	4.7 ± 0.53
10^6	44 ± 6.0	96 ± 5.5	6.1 ± 0.36
10^7	56 ± 5.2	85 ± 6.9	6.3 ± 0.51
10^8	95 ± 10.1	153 ± 11.1	6.5 ± 0.33
10^9	120 ± 7.9	196 ± 8.8	7.1 ± 0.21
10^{10}	144 ± 10.4	216 ± 13.2	6.8 ± 0.28

Table 5.8: Survival curve parameters resulting from the cold-shock of cultures of varying cell densities. Data is derived from Figure 5.10

As prolonged survival occurs in a density-dependent manner, it appears that stationary phase cells may possess factors promoting survival. Such factors are not proteins synthesised as a result of cold-shock (Section 5.13). *C. jejuni* does not possess a RpoS homologue (Section 7.1) and it is not known how stationary phase proteins are regulated in *C. jejuni*. Extrapolation to the poultry industry, indicates that the possibility of plateable *C. jejuni* cells surviving on the refrigerated product, long enough to be ingested, depends on the initial density, and hence the effectiveness of decontamination measures.

5.9 Effect of strain variation on survival in the plateable and non-plateable states

The survival of four *C. jejuni* and two *C. coli* strains was examined at 4°C under microaerobic atmosphere in both the plateable state (Figure 5.15) and the non-plateable state (Table 5.9).

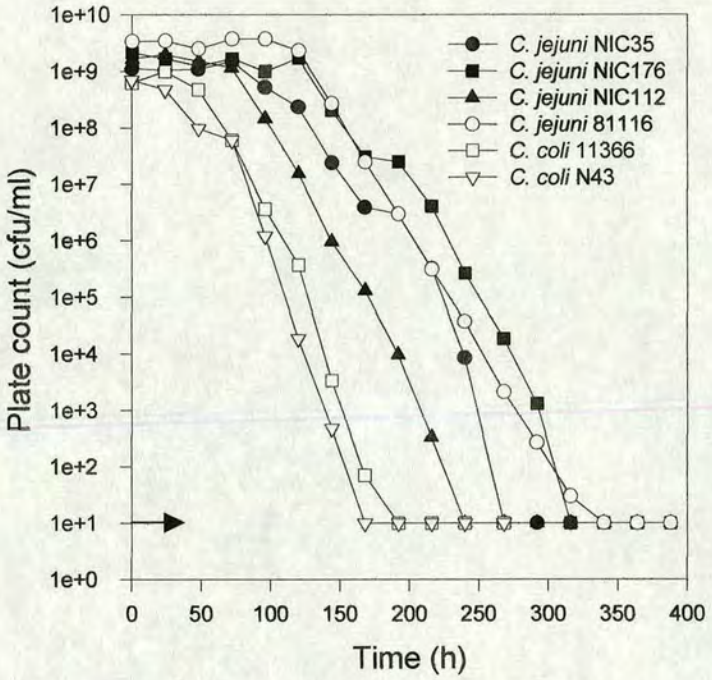


Figure 5.15: Comparison of the survival of various *Campylobacter jejuni* and *Campylobacter coli* strains in the plateable state under microaerobic atmosphere at 4°C. Arrow indicates the detection limit (10 cfu/ml). Mean of two separate experiments

All species and strains exhibited considerable survival in the plateable state, although there was a large degree of strain variation (range: 7-14 days). *C. jejuni* strains survived refrigeration better than *C. coli* strains, exhibited by a prolonged plateau phase (102 ± 13h compared to 48 ± 4h) and a slight decrease in the decline rate (7.14 ± 0.4h and 5.7 ± 0.8h respectively). The potential of the non-plateable, spiral forms of these strains to resuscitate was examined according to Section 5.3 (Table 5.9).

Dilution	Neat	-1	-2	-3	-3 spent	-4	-5	-6
C. jejuni NIC35	0/5	5/5	5/5	5/5	0/5	5/5	5/5	5/5
C. jejuni NIC176	0/5	5/5	5/5	5/5	0/5	5/5	5/5	3/5
C. jejuni NIC112	0/5	5/5	5/5	5/5	0/5	5/5	5/5	2/5
C. jejuni 81116	0/5	5/5	5/5	5/5	0/5	5/5	5/5	5/5
C. coli 11366	0/5	5/5	5/5	5/5	0/5	5/5	5/5	5/5
C. coli N43	0/5	5/5	5/5	5/5	0/5	5/5	5/5	5/5

Table 5.9: Effect of resuscitation via dilution into fresh or spent medium and temperature upshift from 4 to 37°C on different *Campylobacter jejuni* and *Campylobacter coli* strains

All of the strains could be resuscitated after two days entry into the non-plateable state at a dilution of 10^6-fold, which excludes the presence of plateable cells. Furthermore, each strain could not be resuscitated upon incubation in spent medium, indicating the presence of an inhibitory factor within the spent medium. Therefore, resuscitation of the non-plateable spirals has been achieved in six strains covering the two major thermophilic *Campylobacter* species causing enteric disease in humans.

These strains were only two days into the non-plateable state. Using animal models and egg passage, it was possible to resuscitate cells between 13-28d into the non-plateable state (Jones *et al.*, 1991a, Stern *et al.*, 1994, and Cappelier *et al.*, 1999a,b), in a strain dependent manner (Cappelier & Federghi, 1998). Other groups have failed to resuscitate non-plateable cells (Medema *et al.*, 1992, van de Giessen *et al.*, 1996 and Fearnley *et al.*, 1996). Assuming the progressive injury theory, differences may lie in the relative times required for strains to enter the proposed moribund state. The speed of entry into any moribund state would depend on incubation conditions, and may well be species/ strain dependent.

5.10 Effect of cold-shock at 4°C on the survival of *Campylobacter jejuni* 81116 grown in ABCD broth

As performed previously with Brucella-FBP broth (Section 5.2), cells were grown to mid-exponential phase in ABCD broth, before subjection to cold-shock at 4°C and monitoring of the cells physiological status (Figure 5.16).

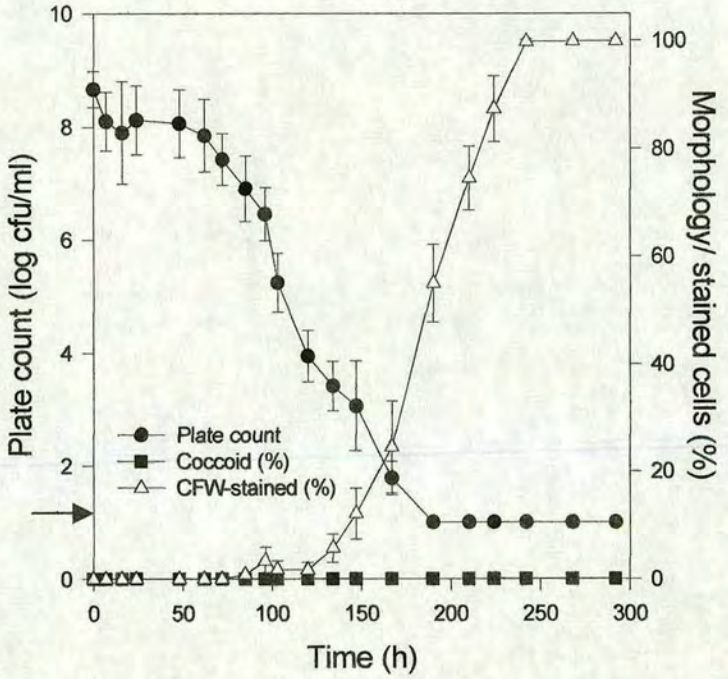


Figure 5.16: Effect of cold-shock at 4°C on *Campylobacter jejuni* 81116 cells grown in ABCD broth. Arrow indicates the detection limit (10 cfu/ml). Mean of three separate experiments

The cells survived for 167h (~7 days) in the plateable state, consisting of a 48h plateau phase and a 119h (~5 days) decline phase with a decline rate of 6.05 ± 0.31h. During the experiment the cells were spiral in morphology. The population began to lose the ability to exclude CFW after 150h (6.25 days), just prior to entry into the non-plateable state, unlike the situation in Brucella broth, where the ability to exclude CFW is lost after entry into the non-plateable state (Section 5.2).

Cells survived refrigeration longer in Brucella broth (~250h; Figure 5.14) than in ABCD broth (~180h) at a density of 10^8 cfu/ml, perhaps due to differences in nutrient compositions between the two broths and the ability of *C. jejuni* to utilise them for maintenance of the plateable state.

The potential of two day old non-plateable cells to resuscitate was examined as described previously (Section 5.3), by dilution into fresh ABCD broth and temperature upshift to 37°C (Table 5.10).

ilution	Neat	-1	-2	-3	-3 spent	-4	-5	-6	-7	-8
. tubes urbid	0/5	5/5	5/5	5/5	0/5	5/5	5/5	4/5	0/5	0/5

Table 5.10: Resuscitation of cold-shocked, non-plateable *Campylobacter jejuni* 81116 cells grown in ABCD broth. Mean of two separate experiments. Total count in undiluted sample = 8.7×10^8 cells/ml

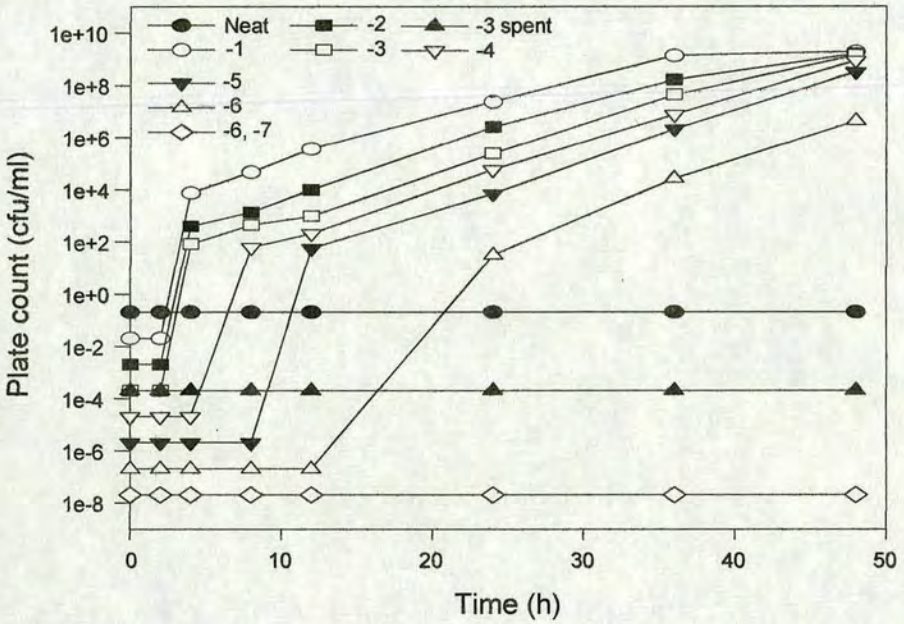


Figure 5.17: Resuscitation of cold-shocked, non-plateable *Campylobacter jejuni* 81116 cells incubated in ABCD broth. Limit of detection is 10 cfu/ml. Mean of two separate experiments

Growth was observed up to the 10^6-fold dilution. This indicated resuscitation of non-plateable cells, because statistically less than 2×10^{-7} plateable cells were present in a total population of 870 cells/ml (making 1740 cells per two mls resuscitation medium). Analysis by the MPN method revealed that 1.3×10^6 viable cells/ml were present at the onset of resuscitation; hence 0.15% of the total population were capable of resuscitation. Resuscitation does not occur if the non-plateable cells are incubated in spent medium, as observed in Brucella broth, indicating the presence of an inhibitory factor (Section 5.4).

Non-plateable cells resuscitated in fresh ABCD broth, took longer to resuscitate (Figure 5.17 and Table 5.10) than non-plateable cells formed in Brucella

broth (Figure 5.5). This was exemplified by a prolonged lag period, perhaps resulting from a combination of a reduced growth rate in this broth (Section 4.6) and the lower nutrient content of ABCD broth. Resuscitation can occur in 30% (v/v) Brucella broth (Section 5.4). This would seem to indicate that if lack of nutrients is a reason for the extended lag period, then they must be either absent or present in low concentrations in ABCD broth. It is possible that the cells had sustained more serious injury than those in Brucella broth, hence longer lag periods may be required for recovery/ repair processes to occur.

5.11 Effect of gas atmosphere and growth phase on survival of cells at refrigeration (4°C) and room temperature (20°C) in the plateable state

The effect of microaerobic and aerobic gas atmosphere and growth phase on survival in the plateable state at both 4 and 20°C was examined (Figure 5.18).

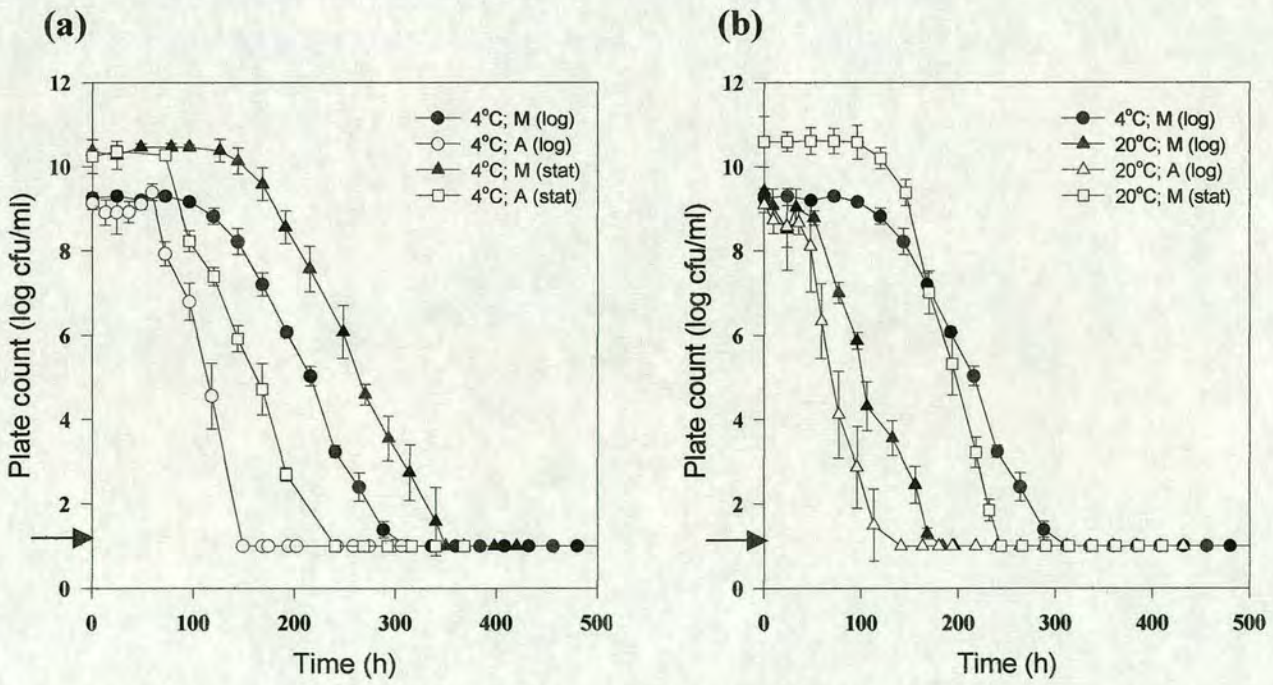


Figure 5.18: Effect of gas atmosphere and growth phase on *Campylobacter jejuni* 81116 survival in the plateable state at (a) 4°C and (b) 20°C; M = microaerobic, A = aerobic; log = exponential phase, stat = stationary phase. Arrow indicates detection limit (10 cfu/ml). Mean of two separate experiments

The survival characteristics of *C. jejuni* under the stresses imposed in Figure 5.18, are shown in Table 5.11. *C. jejuni* 81116 survives longer under microaerobic compared to aerobic conditions (8 days and 13 days respectively [log phase]) at 4°C. Under both gas atmospheres, entry into stationary phase increased survival (Figure 5.14a and Table 5.7). Differences in survival are primarily manifested by increases in the length of the plateau phase, rather than the decline rates. The cells survived for substantial periods of time at refrigeration temperature (8 to 15 days) depending on the physiological state of the cells and the gaseous atmosphere. Even under the most detrimental conditions, cells could be detected for 8 days, within the time-span for consumption of refrigerated poultry.

In comparison to 4°C, survival decreases at 20°C (13 days and 5.5 days respectively [microaerobic, log phase]; Figure 5.18b and Table 5.11). At 20°C, cells incubated under microaerobic compared to aerobic conditions survive longer (5.5 days and 4.5 days respectively [log phase]). Stationary phase affords protection over log phase cells at 20°C (11 days and 5.5 days respectively [microaerobic]).

Incubation condition	Length of plateau phase (days)	Decline rate (hours)	Survival in plateable state (days)	Period of resuscitation (days)
4°C; M (log)	4.2 ± 2.3	6.2 ± 0.3	13 ± 2.4	3.5
4°C; A (log)	3.3 ± 1.1	4.5 ± 0.4	8.0 ± 1.7	2.25
4°C; M (stat)	6.1 ± 1.8	5.6 ± 0.4	15 ± 3.6	3.5
4°C; A (stat)	2.8 ± 0.7	4.3 ± 0.5	9.0 ± 3.1	2.5
20°C; M (log)	1.9 ± 0.7	3 ± 0.3	5.5 ± 1.9	2.5
20°C; A (log)	1.1 ± 0.6	3 ± 0.3	4.5 ± 1.7	1.25
20°C; M (stat)	5.5 ± 2.0	3.25 ± 0.3	11 ± 2.5	2.5

Table 5.11: Comparison of survival curve parameters under various conditions. M = microaerobic, A = aerobic; log = exponential phase, stat = stationary phase. Mean of two separate experiments

In conclusion, incubation temperature and atmosphere, plus the physiological status of the cells affects the length of survival. In broth, *C. jejuni* can survive for substantial periods of time in the plateable state at both refrigeration and room temperatures. At retail the gas atmosphere would vary depending on the packaging conditions used. From these results it is evident that an aerobic atmosphere would reduce *C. jejuni* contamination most efficiently, especially if held at room temperature. Indeed, microaerobic atmospheres may prolong survival.

5.12 Effect of gas atmosphere and growth phase on survival of cells at refrigeration (4°C) and room temperature (20°C) in the non-plateable state

Cells from the experiment in Figure 5.18 were subjected to the resuscitation protocol described in Section 5.3, where non-plateable cells were subjected to temperature upshift and dilution into fresh broth up to 5 days into the non-plateable state (Figure 5.19).

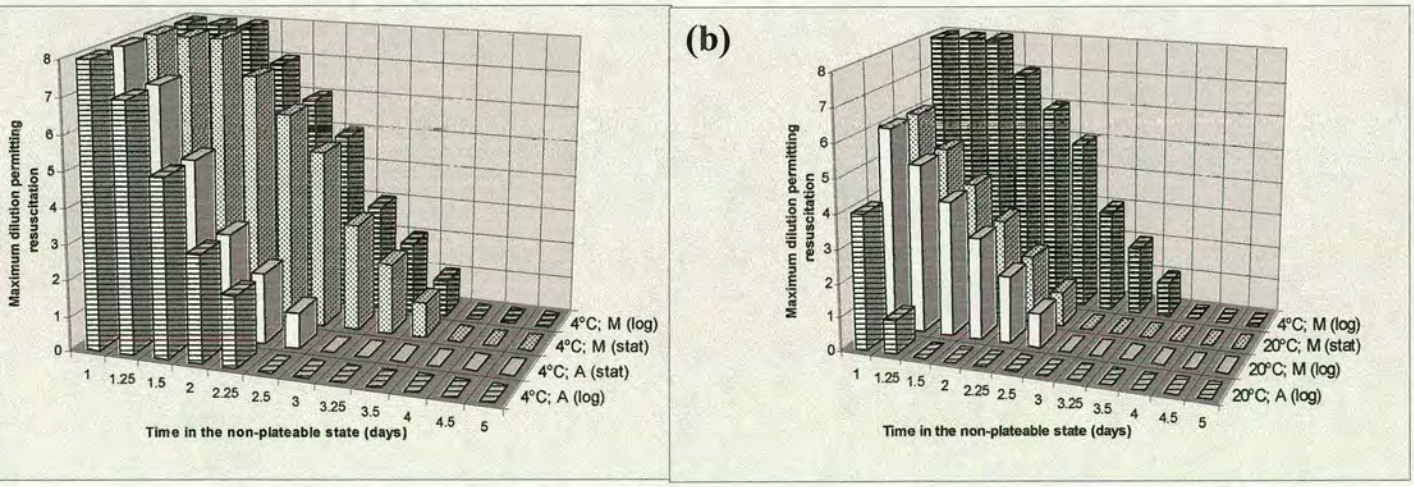


Figure 5.19: Effect of gas atmosphere and physiological growth stage on survival of *Campylobacter jejuni* 81116 in the non-plateable state at (a) 4°C and (b) 20°C. M = microaerobic, A = aerobic; log = exponential phase; stat = stationary phase

Campylobacter jejuni 81116 survives longer in the non-plateable state under microaerobic compared to aerobic conditions, and lower temperatures (4°C) compared to higher temperatures (20°C). However, the growth phase of the cells had no significant effect on the 'window of resuscitation' (Figure 5.19).

The maximum period of resuscitation was only 3.5 days (microaerobic at 4°C; Figure 5.19) under the protocol used, making non-plateable cells unlikely to be a significant reservoir of infection in the environment, however, under specific conditions on poultry surfaces there may be potential for infection from non-culturable forms.

5.13 Effect of cold-shock on *de novo* protein synthesis

One-dimensional PAGE analysis

Many bacteria synthesise specific cold-shock proteins, particularly CspA homologues in response to a reduction in temperature (Section 1.2.3). Cells were grown in ABCD broth supplemented with 0.01% (v/v) Brucella broth to a density of ~5 × 10^8 cells/ml at 37°C under microaerobic atmosphere. The cells were then subjected to a temperature downshift to either 25°C (non-growth permissive) or 32°C (minimum growth temperature) for periods of up to 48h. After subjection to stress, the cells were labelled for 30 min in the presence of 15 μCi/ml [^{35}S]-methionine. The resultant proteins were resolved by 1D-PAGE (Figure 5.20).

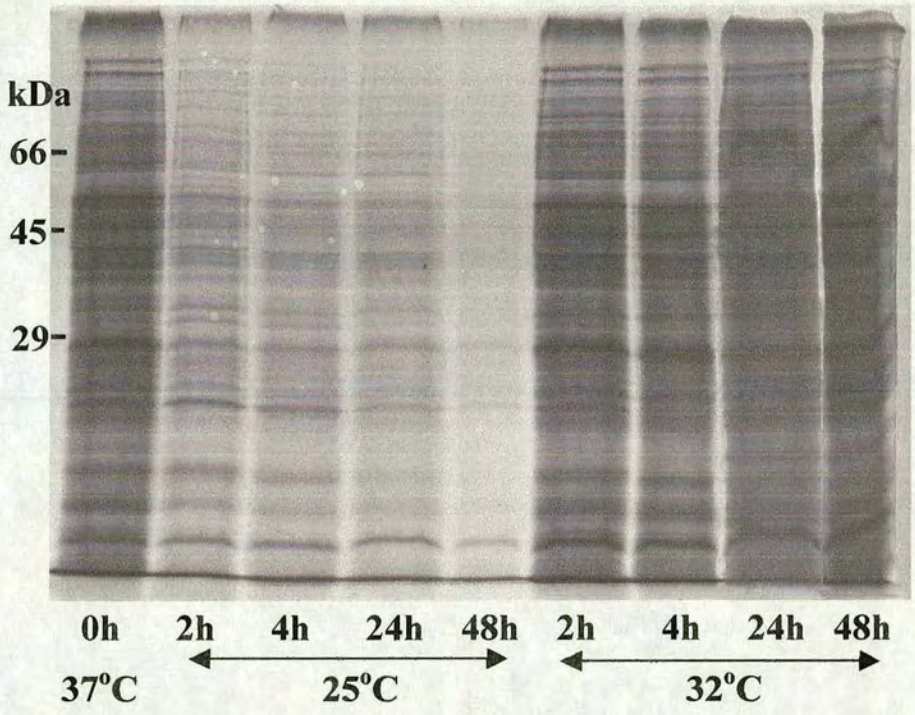


Figure 5.20: Effect of incubation temperature on *de novo* protein synthesis of *Campylobacter jejuni* 81116 over a period of 48h. Each lane possesses 10 μg protein

At the minimum growth temperature (32°C), protein synthesis was maintained over 48h. In contrast, a temperature downshift to 25°C produced a decrease in *de novo* protein synthesis over the 48h stress period. These results indicate that protein synthesis is occurring at these temperatures; therefore the possibility of the production of specific cold-induced proteins exists. No proteins induced as a result of temperature downshift could be observed by 1D-PAGE. However, this may be due to the comparatively low resolution of the method in comparison to 2D-PAGE, which separates on the basis of both isoelectric point and molecular weight of the protein (O'Farrell, 1975).

Two-dimensional PAGE analysis

When some organisms (e.g. *E. coli* and *S. typhimurium*) are subjected to temperatures below their optimum growth temperature, a cold-shock response is initiated, involving a general reduction in protein synthesis and induction of a small number of 'stress' proteins (e.g. CspA) which adapt the cell for survival (Jones & Inouye, 1994; Section 1.2.3).

Two-dimensional PAGE is a powerful analytical tool when combined with pulse-labelling, for studying adaptive stress responses (Östling *et al.*, 1997). Two-dimensional PAGE analysis has been published in three studies with respect to *C. jejuni* stress responses (Hazeleger *et al.*, 1995, Konkel *et al.*, 1998 and Lázaro *et al.*, 1999). Only one used pulse labelling to determine alterations in *de novo* protein synthesis induced as a result of heat-shock (Konkel *et al.*, 1998). This study would complement the aforementioned study with data concerning the cold-shock response of *C. jejuni*. Cells were stressed for a period of 96h at 32°C, 25°C, or 4°C. Metabolic labelling was performed at various sampling times. The protein samples were prepared and 10 µg protein was loaded per gel. The protein profiles were examined using 2D-PAGE as described in Section 2.10.3. The protein profiles obtained for *de novo* protein synthesis are shown in Figures 5.21.1 to 5.21.3.

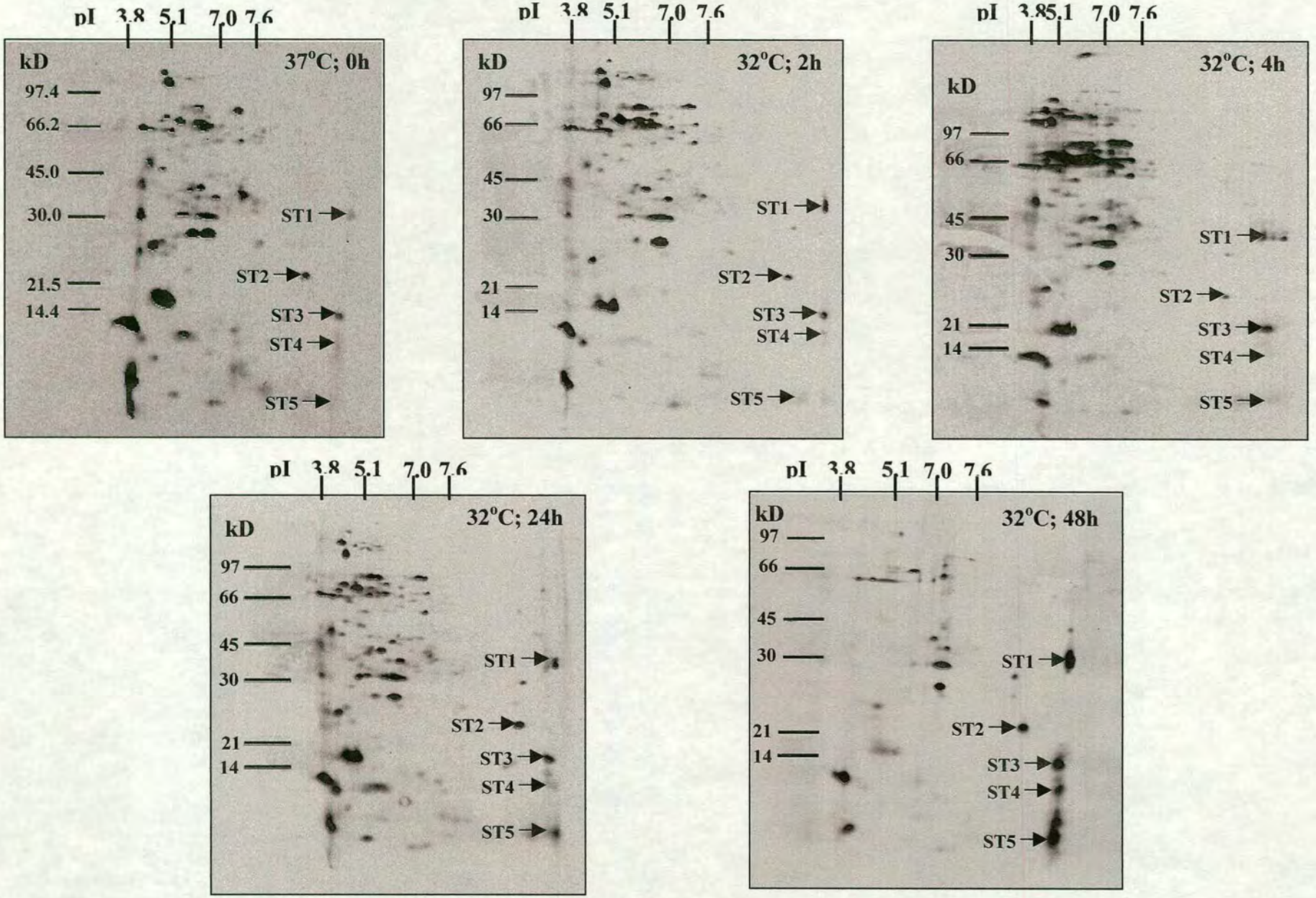


Figure 5.21.1: Autoradiographs of two-dimensional protein profiles of *Campylobacter jejuni* 81116 cells subjected to a temperature downshift from 37°C to 32°C (minimum growth temperature) over a 48h period. Molecular weight in kilodaltons (kDa); pI = isoelectric point

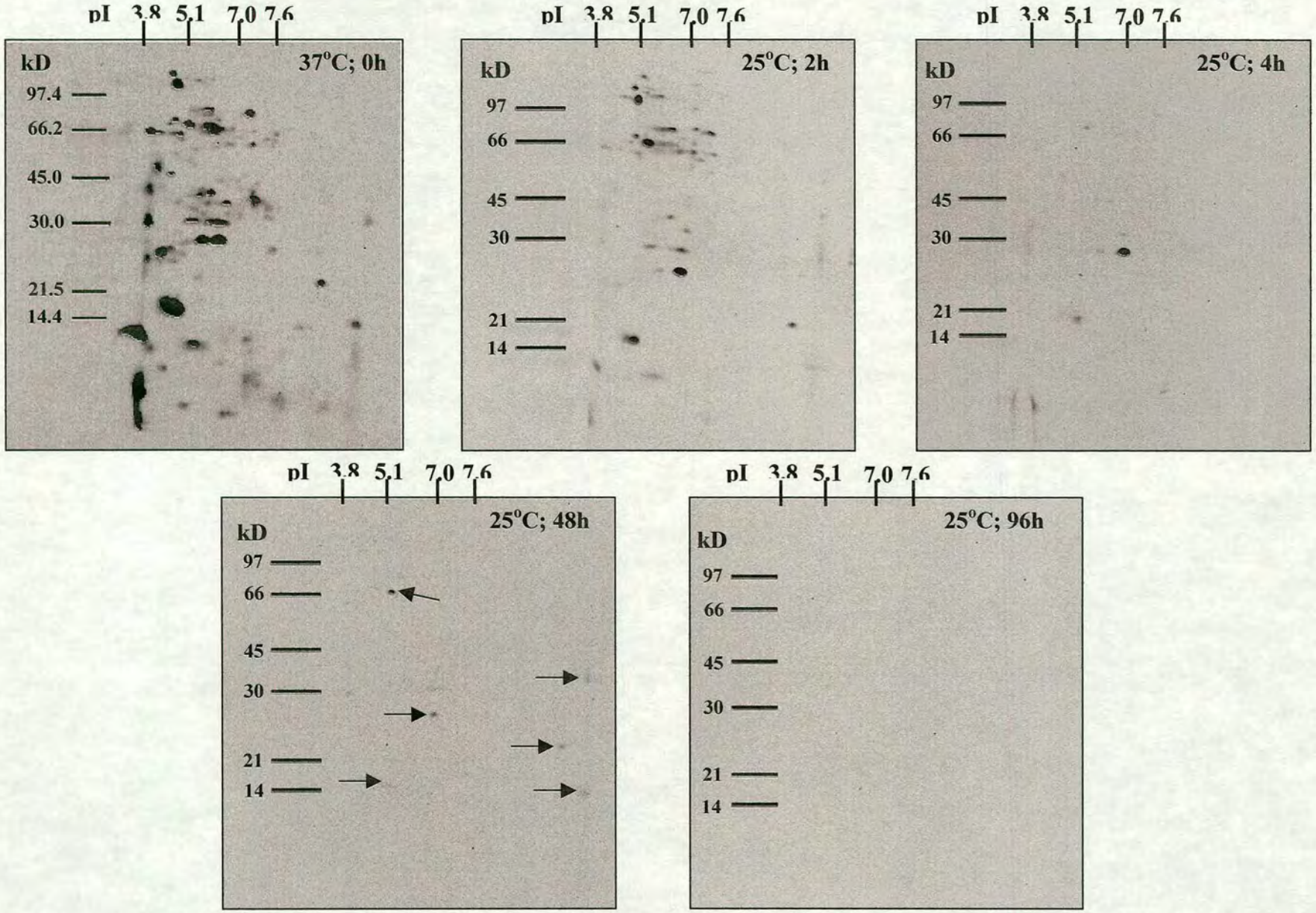


Figure 5.21.2: Autoradiographs of two-dimensional protein profiles of *Campylobacter jejuni* 81116 cells subjected to a temperature downshift from 37°C to 25°C (non-growth permissive temperature; room temperature) over a 96h period. Molecular weight in kilodaltons (kDa); pI = isoelectric point

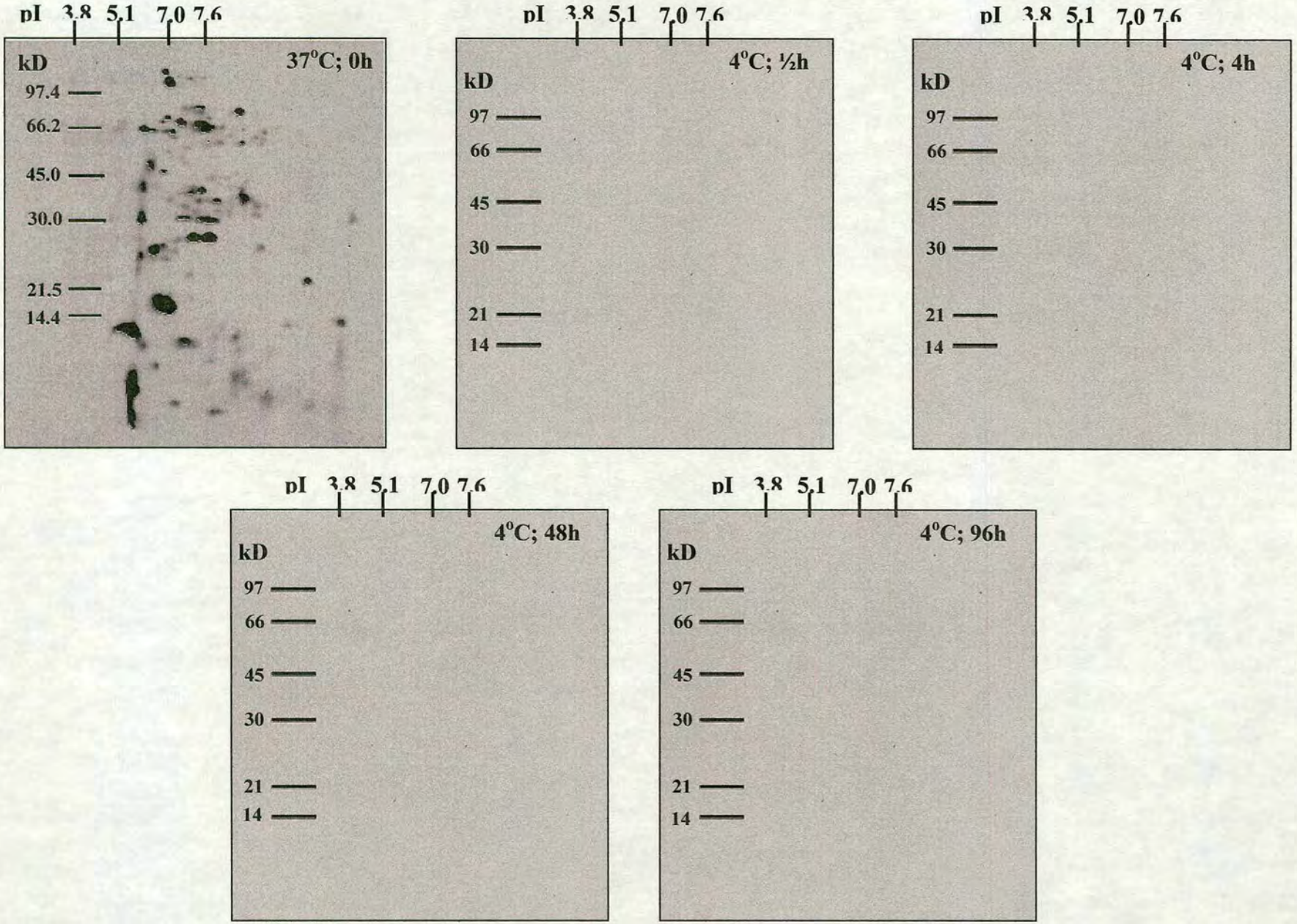


Figure 5.21.3: Autoradiographs of two-dimensional protein profiles of *Campylobacter jejuni* 81116 cells subjected to a temperature downshift from 37°C to 4°C (non-growth permissive temperature; refrigeration temperature) over a 96h period. Molecular weight in kilodaltons (kDa); pI = isoelectric point

When the temperature was decreased from 37 to 32°C (minimum growth temperature) for 4h, no change in the pattern of protein synthesis was observed. After 24h and 48h post-stress, a few specific proteins (ST1-5) were upshifted in synthesis from the levels observed at 37°C (Figure 5.21.1 and Table 5.12). As *C. jejuni* can still multiply at 32°C (growth rate = 146 ± 5.9 min compared to 47.6 ± 2.6 min at 37°C), it is proposed that these proteins were increased as an adaptation to entry into stationary phase at this temperature (Figure 5.22). These proteins are highly basic and may represent DNA binding proteins important in regulating entry into stationary phase.

Protein	Mol. wt (kDa)	pI	Volume of spot					Induction (-fold)
			0h	2h	4h	24h	48h	
ST1	32.4	9.9	306	797	586	1108	2361	7.7
ST2	21.9	8.7	789	762	509	834	1816	2.3
ST3	17.5	9.6	765	771	1761	1310	1352	1.8
ST4	13.8	9.8	96	113	82	423	1218	10.8
ST5	10.6	9.8	52	56	148	748	2607	46.6

Table 5.22: Proteins upshifted upon entry of *Campylobacter jejuni* 81116 into stationary phase at 32°C under microaerobic conditions.

In contrast, a temperature decrease from 37 to 25°C (non-growth permissive temperature) resulted in a large decrease in *de novo* protein synthesis after 2h, with a further reduction 4h post-stress. After 48h stress, only six proteins were detected all of which were present at 37°C. No proteins were synthesised after 96h post-stress (Figure 5.21.2). Therefore, despite the culture becoming 31.7 ± 8.2% coccoid after 48h at 25°C, no specific proteins were synthesised (Figure 5.22).

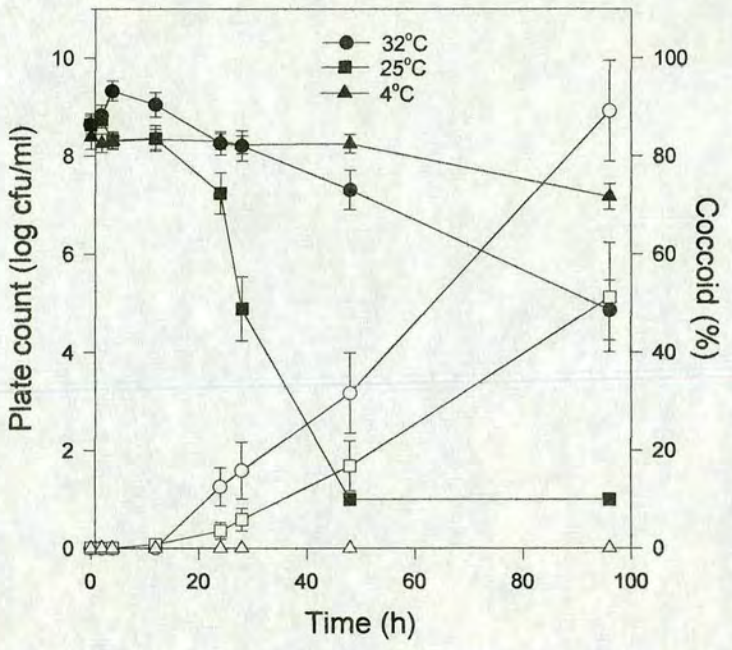


Figure 5.22: Effect of incubation temperature on plating ability and rate of coccoid transformation in *Campylobacter jejuni* 81116 under microaerobic conditions in ABCD broth. Closed symbols = plate counts (cfu/ml), Open symbols = percentage coccoid cells. Mean of two separate experiments.

A temperature decrease from 37 to 4°C (non-growth permissive temperature) resulted in a rapid decrease in *de novo* protein synthesis with no proteins being synthesised 30 min post-stress or at any time up to 96h post-stress (Figure 5.21.3). Over the duration of the experiment the cells were spiral in morphology. It is possible that differences in morphology at 32, 25 and 4°C are due to the increased synthesis of proteins involved in the transition from the spiral to the coccoid state at higher temperatures, such as cell wall modifying enzymes (e.g. PBPs). This could be investigated by sequencing proteins unique to (or absent in) coccoid cells compared to spiral cells. Recently, differences in PBP levels between spiral and aged, coccoid forms of *H. pylori* have been observed (DeLoney & Schiller, 1999). Differences in protein composition between spiral and coccoid forms of *Campylobacter* species have not been observed via 1D-PAGE (Jacobs *et al.*, 1993 and Shibata *et al.*, 1992),

however 2D-PAGE may permit greater resolution of differences. Differences between the two morphological states was not observed in a study by Hazeleger *et al.* (1995), however, only total cellular protein was examined rather than *de novo* protein synthesis. It should also be noted that differences in protein activity (such as cell wall modification enzymes) under stress may result in the formation of the coccoid state in the absence of *de novo* protein synthesis. Such activity would probably be temperature-dependent, with slower rates at lower temperatures. This is supported by the present data and literature (Figure 5.1; Hazeleger *et al.*, 1995 and Lázaro *et al.*, 1999).

At all temperatures examined, no cold-specific proteins were induced, unlike the situation observed in *E. coli* (Jones & Inouye, 1994) and *S. typhimurium* (N. Holden *et al.*, unpublished). Screening of the newly sequenced *C. jejuni* genome (Sanger Centre; Section 7.5) for CspA and CspB homologues was unsuccessful. *C. jejuni* does not produce a cold-shock response as typified in some other mesophiles. However, comparison of the plate counts and protein synthesis profiles at 4°C, indicates that the cells can survive perfectly well in the absence of protein synthesis. How can such a situation arise? It appears that cells are entering a 'stasis' of reduced metabolic activity induced by low temperature. This is evident from the reduction in oxygen consumption, and ATP consumption at low temperatures (Hazeleger *et al.*, 1998). It is possible that the cells are capable of relying on the proteins and energy sources already present.

Recently, it has been proposed that log phase cells self-destruct due to the uncoupling of catabolism and anabolism caused by inimical processes, resulting in a free radical burst. Log phase cells being more metabolically active produce a greater

free oxygen radical burst than stationary phase cells (Dodd *et al.*, 1997). This may explain the difference in *C. jejuni* survival at 20 and 4°C (Section 5.1). It could be envisaged that in the absence of a 'cold-shock response', *C. jejuni* cells incubated at higher non-growth permissive temperatures (20-25°C) will have increased metabolic activity compared to cells incubated at lower temperatures (4°C), hence a greater free oxygen radical burst would be produced, and survival accordingly reduced. Evidence supporting this theory can be derived from the temperature-dependent differences in the rates of coccoid transformation and loss of plating ability (Figure 5.1), rates of reduction in *de novo* protein synthesis (Figures 5.21.1-5.21.3), and the rates of oxygen consumption, ATP synthesis, and catalase activity (Hazeleger *et al.*, 1998). This could be examined by adding paraquat to cells incubated at different temperatures and observing survival in the presence and absence of free radical scavenging enzymes such as superoxide dismutase and catalase.

5.14 Summary

C. jejuni can survive at both refrigeration temperature (c. 4-8°C) and room temperature (c. 20°C) for periods of time sufficient to pose a health risk (Section 5.11). The length of survival is dependent on the incubation temperature (Figure 5.1), gas atmosphere (Figure 5.18), cell density (Figure 5.14) and strain variation (Figure 5.15).

The rate of coccoid transformation and loss of plating ability increases as the incubation temperature increases, revealing the presence of a non-plateable, spiral state in the transformation process (Section 5.1). The non-plateable cells possess metabolic activity, as indicated by the ability to exclude CFW and PI, reduce INT and hydrolyse CFDA (Section 5.7). Metabolically active non-plateable cells can be

resuscitated by a temperature upshift and dilution into fresh broth (Section 5.3). Survival in the non-plateable state occurs over a short period (up to 4d) and is affected by incubation temperature and gas atmosphere.

Entry into the non-plateable state is manifested by a succession of cellular injuries. Initially, cells lose the ability to be cultured on selective agar (Section 5.12), followed by outer membrane damage, and then cytoplasmic membrane damage, indicated by loss of the ability to exclude CFW and PI respectively (Section 5.2 and 5.7). Resuscitation of cold-shocked, non-plateable cells is dependent on a temperature upshift to at least 37°C and the presence of nutrients, in conjunction with dilution of the inhibitory medium (Section 5.4). Cold-shocked *C. jejuni* cells are prevented from resuscitating from the non-plateable state in the spent medium by the presence of a 3-10 kDa, acid labile, heat labile, and proteinaceous inhibitor. The inhibitor appears to preferentially affect non-plateable cells (Section 5.4).

C. jejuni does not possess a cold-shock response (unlike *E. coli* and *Salmonella*), exemplified by the absence of the *de novo* synthesis of specific stress proteins at 4, 25 and 32°C (Section 5.13).

Section 6

Effect of disinfectant procedures on the survival of *Campylobacter jejuni*

6.0 Results and discussion: Effect of disinfectant procedures on the survival of *Campylobacter jejuni* 81116

Hydrogen peroxide (H_2O_2) and trisodium phosphate (TSP; Na_3PO_4; M_r = 163.94) have both been used in the poultry industry at concentrations of 200 mM and 610 mM respectively (Hwang & Beuchat, 1995). It is believed that TSP is bactericidal due to the high alkalinity of the solutions (Li *et al.*, 1995), however, it is possible that TSP also acts via pH, osmotic stress or a combination of both stresses. The concentrations of TSP used, and their respective pH are indicated in Table 6.1 and Figure 6.1. H_2O_2 imposes an oxidative shock and campylobacters are susceptible to this stress.

mM	% (w/v)	pH	Lethal/ Sublethal
4.6 †	0.075	9.9	Lethal
30.5	0.5	8.63	Sublethal
32	0.525	8.69	Sublethal
33.5	0.55	8.75	Sublethal
46	0.75	9.5	Lethal
61	1.0	10.1	Lethal
305	5.0	11.8	Lethal
610	10.0	12.6	Lethal

Table 6.1: Concentrations of TSP solutions in Brucella broth and their corresponding pH. † = incubated in PBS

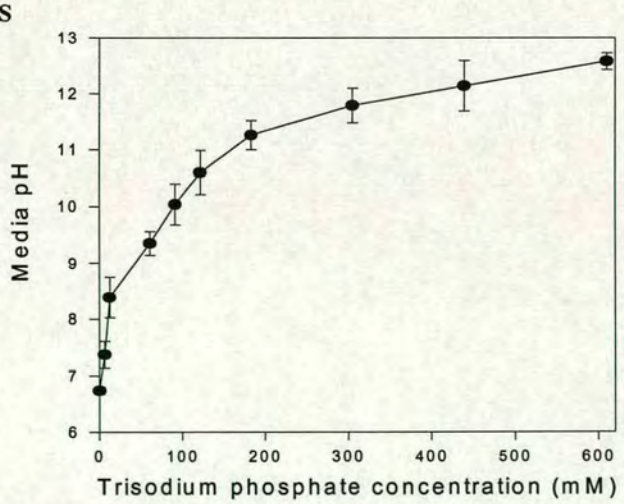


Figure 6.1: Effect of TSP concentration on culture medium pH

As observed in Figure 6.1, a 610 mM working solution provides a pH of 12.6. However, as the TSP concentration increases, not only does the pH of the solution increase, but the ionic composition changes, therefore increasing osmolarity. In particular the Na^+ ion concentration will increase.

An experiment was conducted to determine whether the bactericidal effect of TSP was due to increased pH or an increase in ionic strength of the solution. Exponential phase cells were examined for their ability to survive over 30 mins in Brucella broth containing either 61 mM TSP or 305 mM TSP, which was either left unbuffered, or buffered at pH 6 or pH 7 with orthophosphoric acid (Figure 6.2). It was calculated that a 61 mM or 305 mM TSP solution would contain 46 mM or 229 mM Na^+ ions respectively.

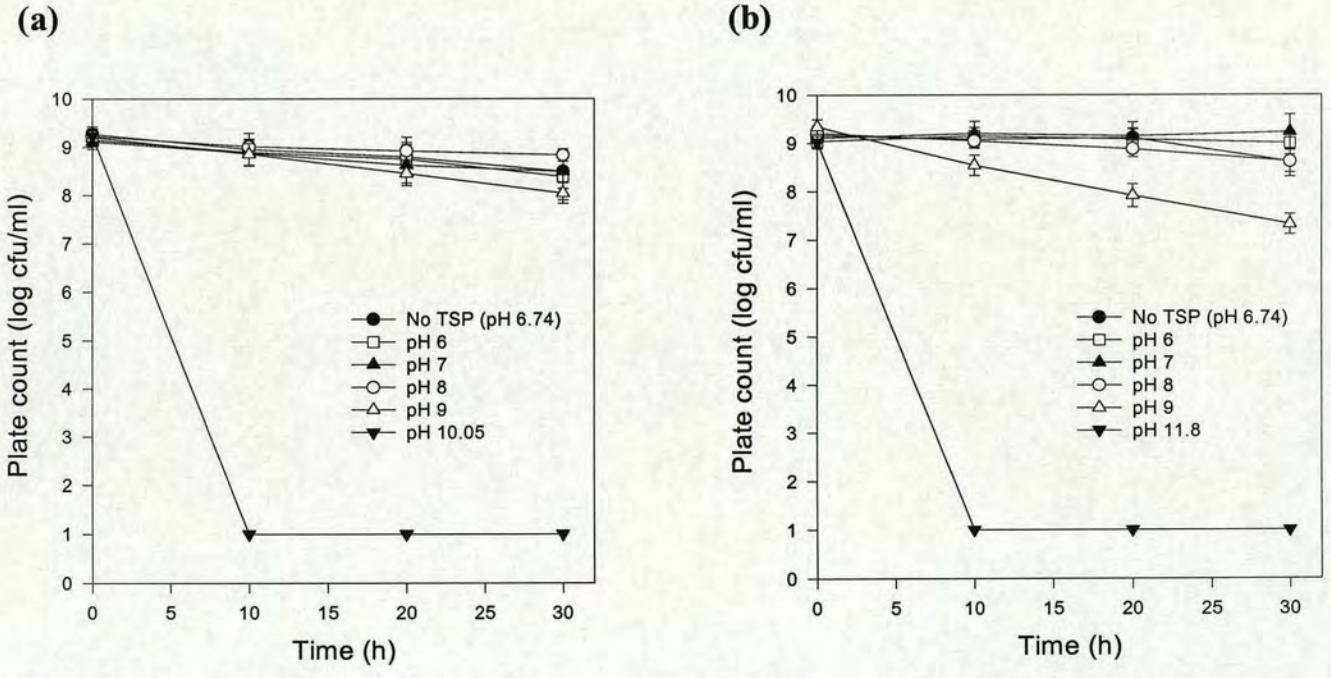


Figure 6.2: Effect of TSP with respect to increased sodium concentration and pH on the survival of *Campylobacter jejuni* 81116 (a) 61 mM TSP and (b) 305 mM TSP. Mean of two separate experiments

In the unbuffered systems at concentrations of both 61 mM and 305 mM TSP, no plateable cells were detected after 10 min incubation. In contrast, buffering of these TSP concentrations at either pH 6, 7 or 8 resulted in no significant loss of plating ability over the duration of the experiment. However, at pH 9, cells incubated in the presence of 305 mM TSP lost plating ability more rapidly than cells incubated in 61 mM TSP, differing in survival by an order of magnitude after 30 min incubation. These results indicate that an increase in alkalinity is the major reason for bactericidal action of TSP, however an increase in ionic concentration does exert an effect.

In order to elucidate further the mechanism of action of TSP, survival of exponential phase cells was examined in various buffers over the pH range 7-10 (Figure 6.3). Some systems possessed Na^+ ions (TSP, carbonate), or K^+ ions (K_2PO_4), whilst others possessed neither (Tris-HCl). The solutions containing 61 mM TSP and 100 mM K_2PO_4 were buffered using orthophosphoric acid. No pH changes were observed after experimentation (30 min), and all buffer systems used were stable over a period of 2 weeks.

Survival over the 30 min stress period was pH-dependent in the case of 61 mM TSP, 100 mM K_2PO_4 and carbonate buffers, with loss of viability only in buffers of pH 9-10. However, it is evident that pH alone is not the cause of loss of viability, because borate and Tris buffered systems at pH 9-10 did not produce a loss of viability. There are differences in the ionic composition of the buffers. The borate and Tris buffers lack the Na^+ or K^+ ions present in the others.

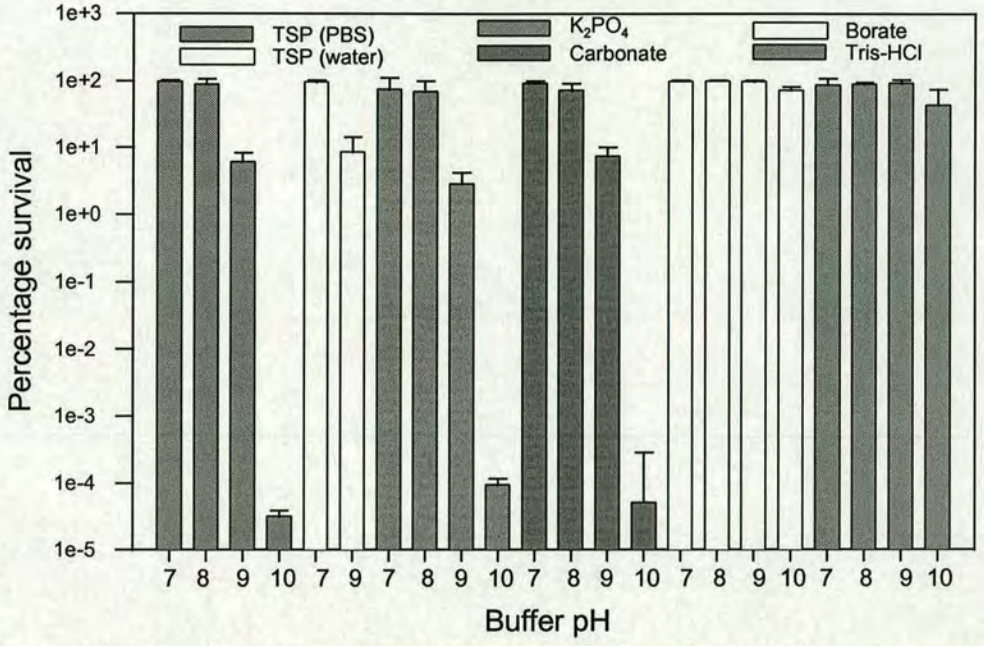


Figure 6.3: Effect of various buffered systems over a pH range of 7.0-10.0 on the survival of *Campylobacter jejuni* 81116 over a 30 min period. Mean of two separate experiments

Therefore the pH dependent loss of viability only occurred in the presence of Na^+ or K^+ ions. However, the evidence suggests that it is a combination of high pH and Na^+ ions that produces the lethal effect because at pH 6-7, survival in the presence of 46-229 mM Na^+ is the same as in the controls (Figure 6.2). It is possible that a system(s) is protecting cells incubated at pH 9-10 in Tris/ borate buffers which is overwhelmed or absent at pH 9-10 in the presence of 61 mM TSP, carbonate or 100 mM K_2PO_4 buffers (Na^+ or K^+ ions). Alternatively the phenomenon may be due to the presence of an active system that only works in the presence of Na^+ ions (e.g. NhaA) or K^+ ions (e.g. KefB).

In other mesophilic organisms, survival at alkaline pH is mediated by the action of Na^+/H^+ antiporters, such as NhaA, which accumulate H^+ ions whilst extruding Na^+ ions. The activity of NhaA increases over the pH range of 6.5-8.5 (Hall *et al.*, 1995). The genome of *C. jejuni* has revealed three homologues of Na^+/H^+ antiporters, NhaA1, NhaA2, and NapA (Section 7.6). It is possible that in the presence

of 61 mM TSP (46 mM Na^+ ions), NhaA is induced/ activated and extruding Na^+ ions over the pH range 7-8, allowing survival of the cells. Between pH 9-10 however, the external Na^+ gradient is too high for the Na^+/H^+ antiporters to operate, and hence survival decreases. A similar situation has been observed in *E. coli* cells (Hall *et al.*, 1995). This only explains the phenomenon observed with Na^+ ions, however, K^+ ions are the only anions present in the K_2PO_4 buffered system. Therefore does *C. jejuni* possess K^+ exporters? Searching the newly sequenced genome revealed the presence of KefB, a glutathione-regulated K^+-exporter (Section 7.7) that may play a similar role to NhaA in maintenance of cellular homeostasis upon alkaline shock in the presence of K^+ ions. This could be investigated by creating genetic knockout mutants of NhaA and KefB, and observing survival at pH of various alkalinity. Additionally, the effect of specific antiporter inhibitors (e.g. amiloride for NhaA) on survival could be examined.

In this thesis, there is evidence that *C. jejuni* possesses an adaptive response to sublethal concentrations of TSP (Section 6.3). This effect is likely to be due to a pH change from 6.7-8.7 because the presence of 32 mM TSP would not impose a severe osmotic upshift. Furthermore, *C. jejuni* 81116 cells are capable of surviving for at least 6h in the presence of 1.8 M NaCl (Robb *et al.*, unpublished). Whether other monovalent cations such as Li^+ or divalent cations such as Ca^{2+} or Mg^{2+} ions produce similar pH-dependent responses to Na^+ and K^+ ions in *C. jejuni* could be examined using buffered systems containing these cations.

6.1 Effect of broth and PBS on the lethal concentration of H_2O_2 and TSP required to kill *Campylobacter jejuni* 81116 cells

For the purpose of stress and adaptation studies, using H_2O_2 and TSP, the lethal concentration required to provide a 3-4 log decrease in plate counts over a 30

min stress period was determined. The experiments were performed in both Brucella broth and PBS (pH 7.3) to determine whether there was a buffering effect with broth.

Concentrations up to 0.1 mM H_2O_2 did not produce a significant decrease in survival of *C. jejuni* (Figure 6.4a), indicating that these concentrations were sublethal, and could be used as adaptive concentrations. Concentrations greater than 0.1 mM resulted in a progressive decrease in survival until at 2.0 mM, no plateable cells could be detected after 30 min stress. The lethal concentration used in subsequent studies is 0.5 mM because it provides a 2-3 log decrease in 30 min, providing a benchmark for comparative survival. Similar results were observed regardless of the medium used, hence Brucella broth does not significantly quench H_2O_2.

TSP concentrations up to 0.37 mM and 33.5 mM in PBS and broth respectively (Figure 6.4b) did not significantly decrease survival, hence these concentrations are considered sublethal. Survival at higher concentrations rapidly decreased survival until no plateable cells were detected (<10 cfu/ml) after 30 min incubation, at concentrations of 6.1 mM and 49 mM TSP in PBS and broth respectively. The lethal concentrations used in subsequent studies were 4.6 mM and 46 mM TSP in PBS and broth respectively, providing a 3-4 log decrease in survival over 30 min. Incubation in broth protected against the lethal effects of TSP, as indicated by the increased concentration required to produce an equivalent reduction in survival compared to PBS. The actual buffering pH is not vastly different in PBS (pH 7.3) and broth (pH 6.7); hence it appears that TSP is quenched by the buffering effect of Brucella broth constituents absent in PBS (pH 7.3).

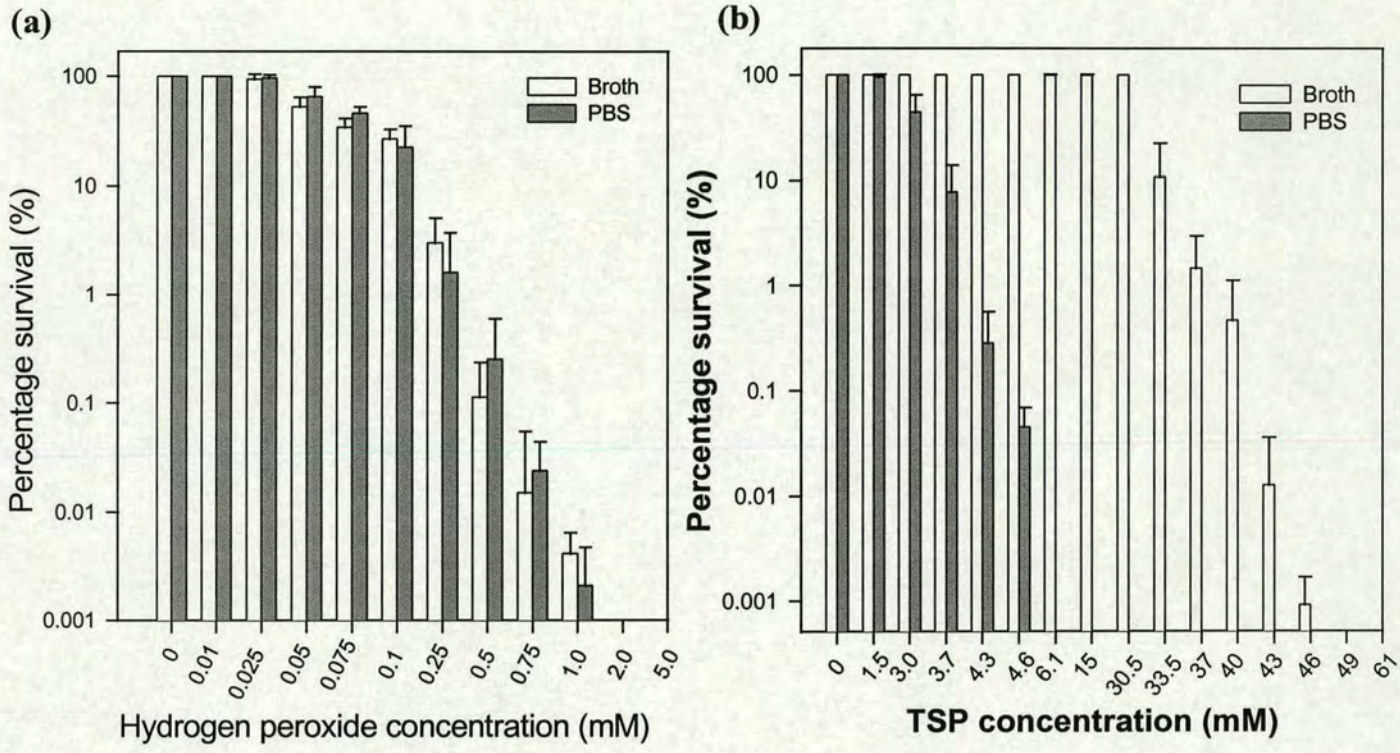


Figure 6.4: Effect of increasing concentration of (a) H_2O_2 and (b) TSP on *Campylobacter jejuni* 81116 survival in Brucella broth and PBS (pH 7.3). Mean of two separate experiments

The effect of 0.5 mM H_2O_2 and 46 mM TSP was examined over a period of 60 min (Figure 6.5).

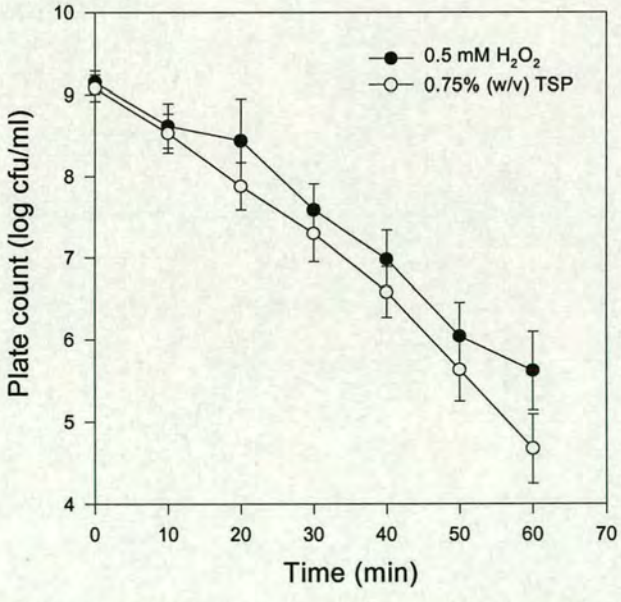


Figure 6.5: Survival kinetics of lethal concentrations of H_2O_2 (0.5 mM) and TSP (46 mM) on *Campylobacter jejuni* 81116 incubated in Brucella broth at 37°C

Survival was better in the presence of 0.5 mM H_2O_2 compared to 46 mM TSP, with decline rates of 5.12 ± 0.26 min and 4.1 ± 0.21 min respectively. D-values were 16 ± 2.1 min and 12.8 ± 1.2 min for 0.5 mM H_2O_2 and 46 mM TSP respectively.

6.2 Effect of incubation temperature on the survival of *Campylobacter jejuni* 81116 to H_2O_2 and TSP

In the poultry industry, low temperatures are often used during the disinfectant washing process and storage to prevent proliferation of food poisoning and spoilage microbes. Previously it has been demonstrated that although proliferation of *C. jejuni* would be prevented, it is possible that substantial survival occurs in the plateable state at low temperature (Section 5.1). To investigate this hypothesis, exponential phase cells were incubated in the presence of lethal concentrations of H_2O_2 or TSP (Figure 6.6).

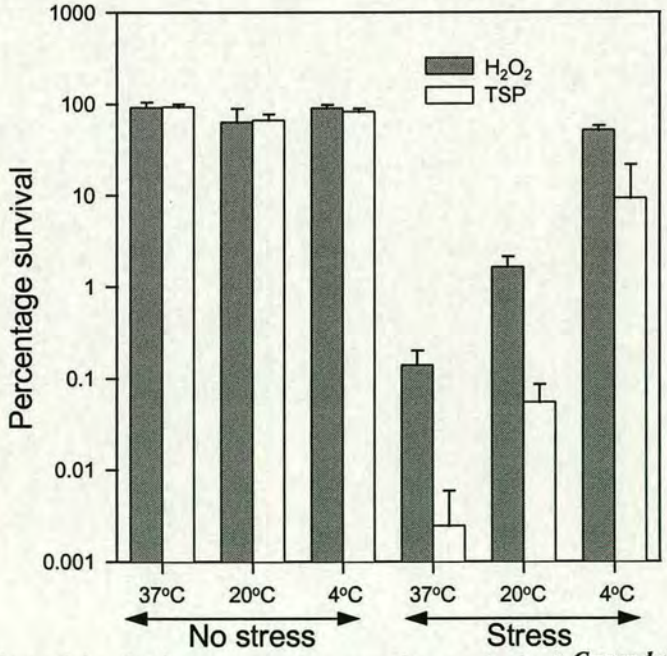


Figure 6.6: Effect of incubation temperature on the survival of *Campylobacter jejuni* 81116 incubated in PBS to 0.5 mM H_2O_2 or 6.1 mM TSP. Cells were incubated for 30 min at the appropriate temperature in the presence of H_2O_2 or TSP. Mean of two separate experiments

As the incubation temperature decreased, the number of cells surviving the lethal stresses increased. Therefore incubation at 4°C affords protection against the deleterious effects of H_2O_2 and TSP experienced at higher temperatures. *C. jejuni* does not have an adaptive response to cold-shock, with the synthesis of specific stress proteins (Section 5.13), hence it is likely that the observed protection is a passive process mediated by reduced metabolic activity. In conclusion, washes utilising these chemicals in the poultry industry would reduce *Campylobacter* levels more efficiently if performed at raised temperatures. Room temperature (~20°C) should be sufficient to prevent excessive proliferation of spoilage organisms, particularly as treatment would only be for short periods (30-60 min maximum).

The effect of incubation at 4°C on *C. jejuni* survival against disinfectant concentrations used during poultry washes was examined over 30 min (Table 6.2).

	No stress			H_2O_2 (200 mM)			TSP (610 mM)		
	4°C	20°C	37°C	4°C	20°C	37°C	4°C	20°C	37°C
Survival (%)	95.8 ± 2.9	65.8 ± 15.6	90.3 ± 5.0	0	0	0	0	0	0

Table 6.2: Effect of incubation at 4°C on survival of *Campylobacter jejuni* 81116 upon subjection to 200 mM H_2O_2 and 610 mM TSP. Mean of two separate experiments

No cells survived at any temperature in the presence of 200 mM H_2O_2 or 610 mM TSP over a 30 min period. This indicates that the disinfectant concentrations used as poultry washes, should be sufficient to reduce *Campylobacter* contamination. The fact that many carcasses are still contaminated at retail, highlights the importance of quenching of the disinfectants by poultry residues, and cross-contamination during poultry processing.

The effect of incubation temperature on a number of lethal stresses (4.6 mM TSP, 0.5 mM H_2O_2, acid (pH 3.8), 684 mM NaCl and 0.13 mM NaOCl) was

examined (Figure 6.7). Experiments were conducted in PBS to eliminate quenching experienced with Brucella broth (Section 6.1). Use of PBS subjects cells to the additional stress of starvation. This will be the case in the poultry industry when disinfectant washes are used. Such washes subject cells to a dual stress; that of the disinfectant and also that of nutrient depletion.

In the presence of all stresses examined, as the incubation temperature decreased, survival in the plateable state was prolonged (Figure 6.7). Decline rates in the presence of all stresses increased as the temperature decreased (Table 6.3). The decline rates were specific for the particular stress, with low temperature particularly prolonging survival against osmotic and oxidative shock mediated by 684 mM NaCl (>140h) and 0.5 mM H_2O_2 (100h) respectively.

Stress	Decline rate (min^{-1})		
	4°C	20°C	37°C
TSP (4.6 mM)	24.5 ± 5.9	9.9 ± 2.1	3.5 ± 1.2
H_2O_2 (0.5 mM)	221.4 ± 21.2	10.0 ± 1.7	3.8 ± 0.9
Acid (pH 3.8)	43.9 ± 5.9	17.2 ± 3.8	12.5 ± 2.6
NaCl (684 mM)	311.6 ± 16.9	27.9 ± 5.3	12.5 ± 3.1
NaOCl (0.13 mM)	12.3 ± 3.9	7.2 ± 2.1	7.2 ± 1.9

Table 6.2: Temperature dependent decline rates of *Campylobacter jejuni* 81116 upon subjection to various chemical stresses. Data derived from Figure 6.7

These results indicate that *C. jejuni* has the potential to survive for short periods (up to 7h) on foodstuffs preserved by acid or salt at room temperature (~20°C). Over such periods a health risk is unlikely, however the possibility of injured or non-plateable cells which can resuscitate has not been addressed, and incubation at 4°C significantly increases survival in the plateable state (Figure 6.7).

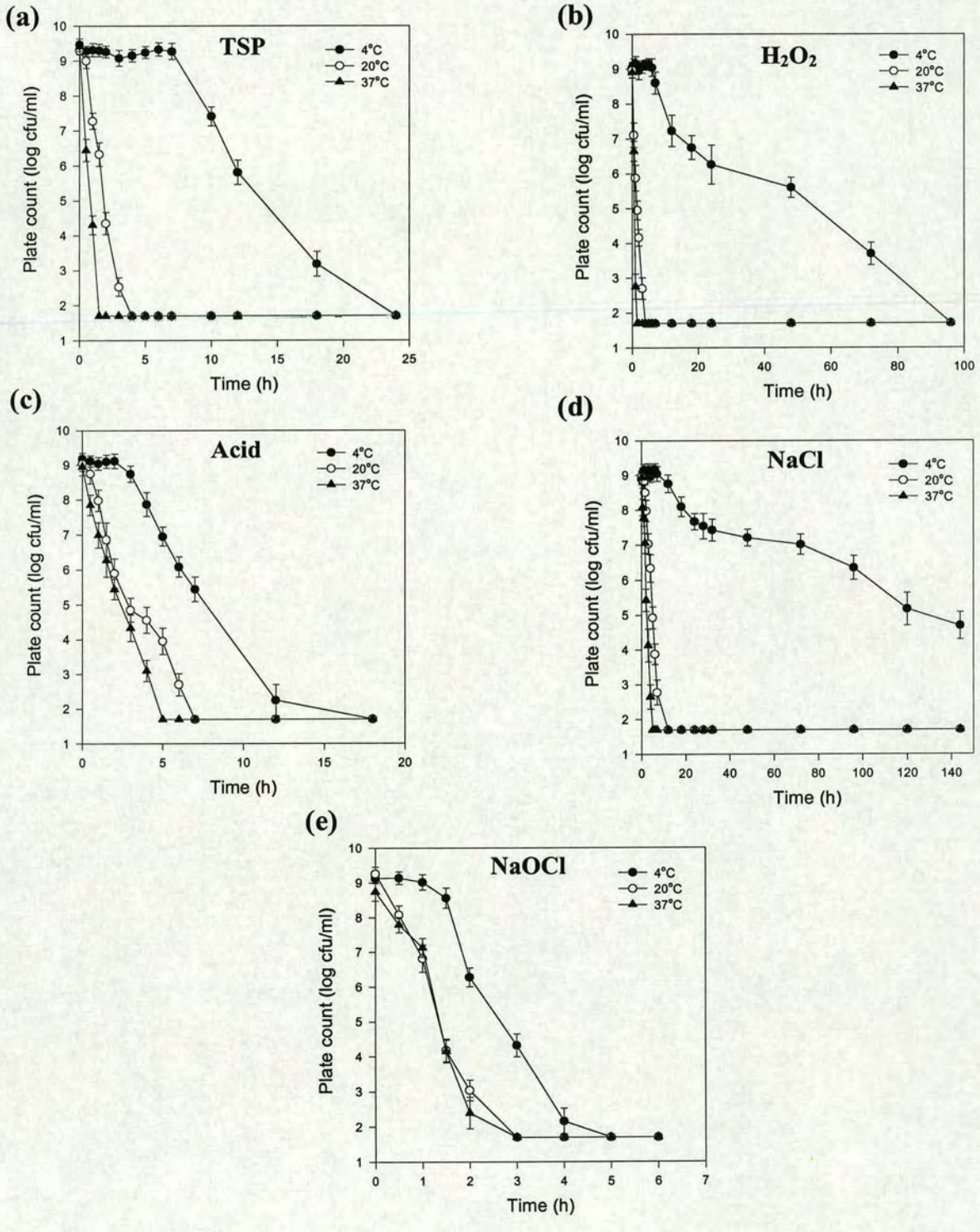


Figure 6.7: Effect of incubation temperature on the survival of *Campylobacter jejuni* 81116 in PBS upon subjection to (a) 4.6 mM TSP, (b) 0.5 mM H_2O_2, (c) acid (pH 3.8), (d) 684 mM NaCl and (e) 0.13 mM NaOCl. Mean of two separate experiments

The most effective stresses at 4°C were NaOCl and TSP, which is encouraging given their usage as disinfectants within the poultry industry. However, a survival shoulder of 2h and 7h with NaOCl (0.13 mM) and TSP (4.6 mM) respectively exists, whereby no decrease in plating ability is observed. Poultry carcasses are dipped in disinfectant for only short periods (30 secs), hence considerable survival may occur, despite reduced bacterial loads and higher disinfectant concentrations. The potential for some cells to resist decontamination procedures exists due to a combination of low temperature (Figures 6.6 and 6.7), quenching of the disinfectant (Figure 6.4), and inaccessibility to the microbe (e.g. under a fold of skin). The potential of injured or non-plateable cells to resuscitate and produce infection (Section 5.3; Cappelier *et al.*, 1999a), coupled with the low infectious dose of the bacterium (Robinson, 1981) should provoke further research into the temperature dependent survival of *C. jejuni*, particularly on the poultry surface.

6.3 Effect of adaptation on survival of *Campylobacter jejuni* 81116 to lethal concentrations of H_2O_2 and TSP

In many bacteria, exposure to sublethal concentrations of a stress promotes survival against lethal concentrations (Foster 1991 and Rocha *et al.*, 1996). Whether exponential phase *C. jejuni* cells could adapt at sublethal concentrations of H_2O_2 and TSP was examined (Figure 6.8).

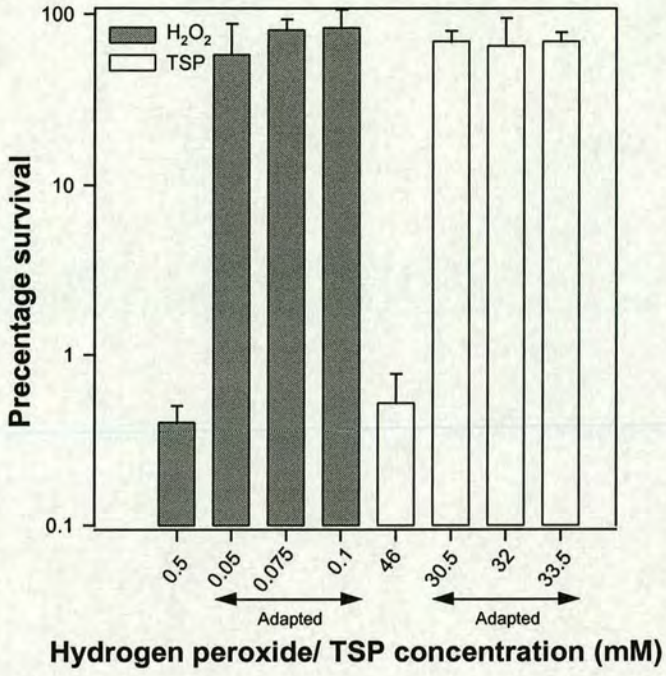


Figure 6.8: Effect of adaptation at sublethal H_2O_2 (0.05-0.1 mM) and TSP (30.5-33.5 mM) concentrations on survival against lethal concentrations. Exponential phase cells were adapted for 60 min prior to challenge with lethal concentrations of H_2O_2 (0.5 mM) or TSP (46 mM) for 30 min. Bars in the legend indicate the nature of the stress applied. Mean of two separate experiments

Adaptation at all sublethal concentrations examined for both H_2O_2 and TSP provided at least a two-log increase in survival against lethal concentrations compared to unadapted cells. Further adaptation experiments used 0.1 mM H_2O_2 and 32 mM TSP as the sublethal concentrations.

Campylobacter cells may experience stresses greater than 46 mM TSP or 0.5 mM H_2O_2. A working solution of TSP used in poultry washes is 610 mM, whilst Campylobacters in environmental situations would routinely experience oxidative shock. The potential to adapt and withstand concentrations in excess of those tested was examined (Figure 6.9).

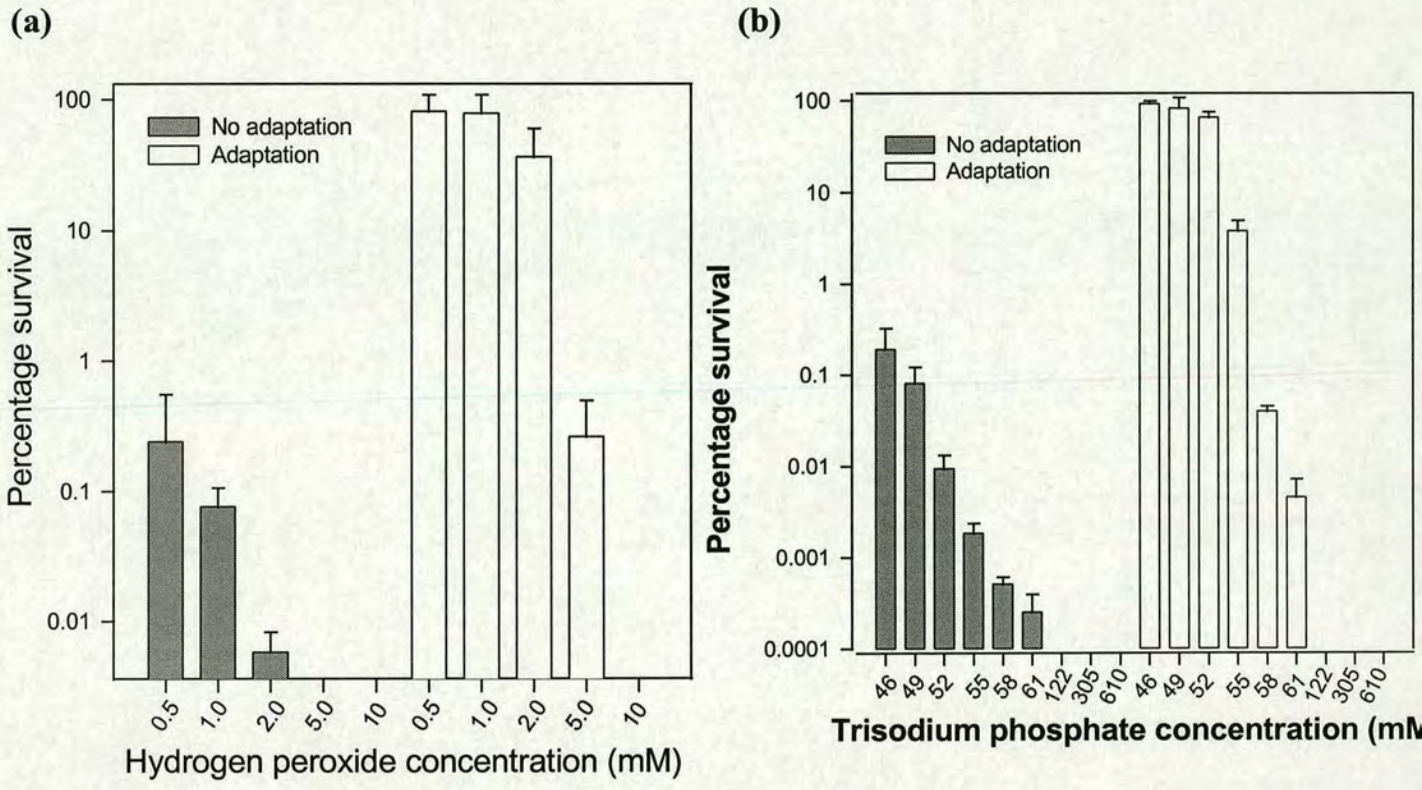


Figure 6.9: Protective effect of adaptation with sublethal concentrations of (a) H_2O_2 (0.1 mM) and (b) TSP (32 mM), against lethal concentrations. Mean of two separate experiments

Adaptation at sublethal concentrations protected against the lethal effects of up to 5.0 mM H_2O_2 and 61 mM TSP respectively. The ability of *C. jejuni* to adapt to oxidative shock and TSP disinfection could contribute to survival during poultry processing. Bacteria hidden beneath folds of skin may experience lower disinfectant concentrations, causing adaptation to substantially higher concentrations. Similarly, quenching of the disinfectant would lead to exposure of the cells to concentrations permitting adaptation.

6.4 Effect of growth phase on survival and adaptation to H_2O_2 and TSP

In most bacteria, stationary phase or starved cells are inherently more resistant to stress compared to exponential phase cells, due to the production of general stress proteins (Huisman *et al.*, 1996; Section 1.2.6). Exponential phase (12h) and stationary phase (24h) cells were examined for their relative resistance to 0.5 mM H_2O_2 or 46 mM TSP in the presence and absence of adaptation (Figure 6.10).

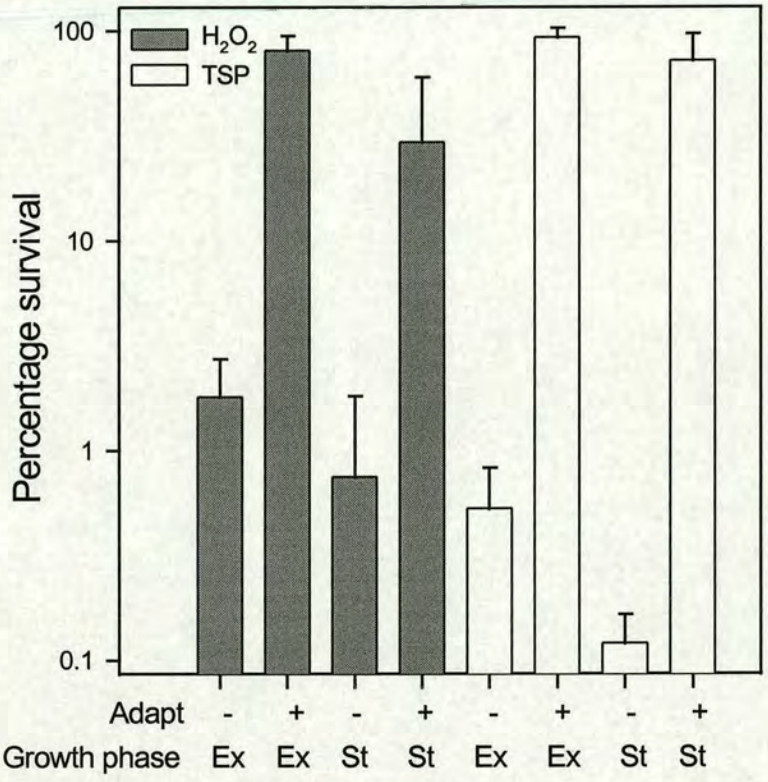


Figure 6.10: Effect of growth phase on the survival of *Campylobacter jejuni* 81116 to lethal concentrations of H_2O_2 (0.5 mM) or TSP (46 mM), in the presence or absence of adaptation with sublethal concentrations of H_2O_2 (0.1 mM) or TSP (32 mM) for 60 min. Ex = exponential phase (12h), St = stationary phase (24h). Mean of two separate experiments

Adaptation of both exponential phase and stationary phase cells to sublethal concentrations provided 100% protection against lethal concentrations of both H_2O_2 and TSP. However, unlike the situation in other bacteria such as *E. coli*, stationary phase cells were not more resistant to the lethal stresses compared to exponential

phase cells. Indeed, in the case of 46 mM TSP, stationary phase cells were ~4.4-fold more sensitive. In many bacteria, stationary phase resistance to stress is mediated by RpoS (Huisman *et al.*, 1996). However, *C. jejuni* does not possess a RpoS homologue (Section 7.1). Therefore *C. jejuni* may possess alternative mechanisms for regulating stationary phase induced gene expression. It would appear that unlike the case with *Salmonella* and *E. coli* cells, stationary phase *C. jejuni* cells do not pose a greater problem in terms of resistance to disinfection than exponential phase cells.

Further research would be beneficial in this area. As already observed (Section 4.0), stationary phase in *C. jejuni* is composed of a range of different cellular forms, their relative ratios governed by the time spent in stationary phase. In particular spiral cells are observed to elongate as stationary phase progresses. A 36h or 48h old culture may have entirely different resistance properties than the 24h stationary phase culture used in this experiment. Such studies would also indicate whether the elongated cells are important with respect to survival outside the host.

6.5 Effect of adaptive cross-protection between H_2O_2 and TSP

The adaptive stress responses elicited by sublethal concentrations of H_2O_2 and TSP were examined to see whether they provided cross-protection against lethal concentrations of the other stress (Figure 6.11).

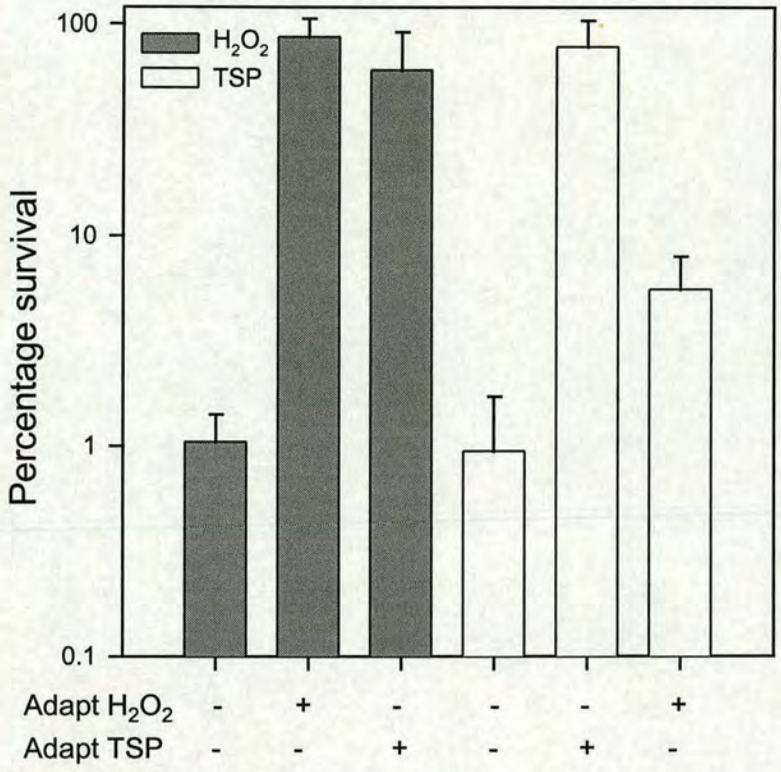


Figure 6.11: Effect of adaptation with sublethal concentrations of H_2O_2 (0.1 mM) or TSP (32 mM) on cross-protection against lethal concentrations of either H_2O_2 (0.5 mM) or TSP (46 mM). Bars in the legend indicate the nature of the lethal stress applied. Mean of two separate experiments

Adaptation with TSP gave a two log increase in cells surviving the lethal stress of 0.5 mM H_2O_2, whilst adaptation with H_2O_2 conferred a one log increase in resistance to a lethal concentration of 46 mM TSP. This indicates that at least some protection mechanisms may be common to both stresses, possibly due to the induction of stress proteins.

Cross-protection has been observed in other bacterial species. For example, in *S. typhimurium*, proteins involved in the protective response against H_2O_2 are induced in response to heat shock, ethanol stress, and UV irradiation (Morgan *et al.*, 1986). The effect of cross-protection is worrying from the perspective of the food industry. Situations may arise where exposure to sublethal concentrations of TSP or H_2O_2 may lead to resistance to lethal concentrations of these stresses. *C. jejuni* is only likely to experience TSP during the washing process in a poultry processing plant. However,

oxidative shock would be experienced by the bacterium upon release into the environment, during processes such as evisceration, hence the cells may be protected against the disinfection procedure due to cross-protection mechanisms induced by mild oxidative shock.

6.6 Effect of chloramphenicol addition on the protection elicited by adaptation with sublethal concentrations

Adaptive responses generally involve the synthesis of proteins which protect the organism from the particular stress (Foster, 1991). This can be examined by addition of chloramphenicol during adaptation to prevent protein synthesis, prior to challenge with the lethal concentration (Figure 6.12). Chloramphenicol is bacteriostatic and prevents protein synthesis. The bacteria are not killed over the duration of chloramphenicol treatment, with 100% survival of cells after 60 min.

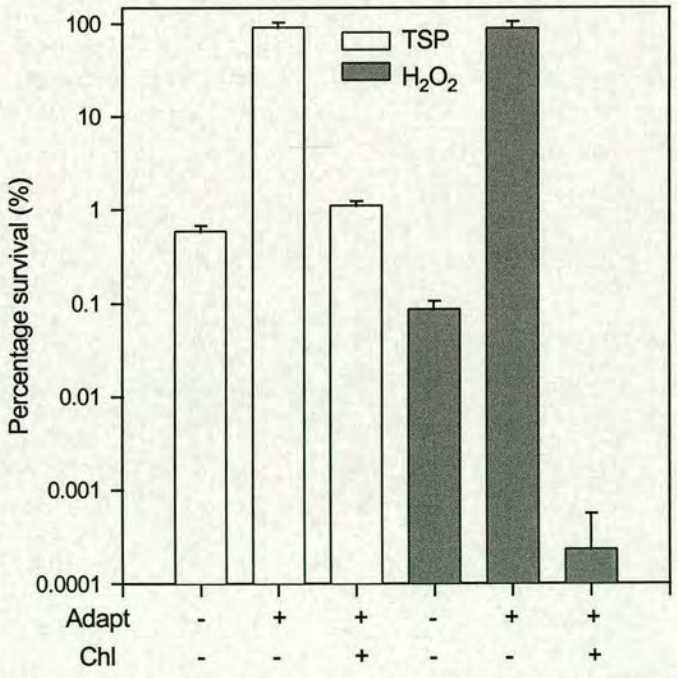


Figure 6.12: Effect of chloramphenicol treatment (100 μg/ml) during adaptation with H_2O_2 (0.1 mM) or TSP (32 mM). Cells were adapted for 60 min in the presence or absence of chloramphenicol, prior to challenge with lethal concentrations of H_2O_2 (0.5 mM) or TSP (46 mM) for 30 min. Chl = chloramphenicol. Mean of two separate experiments

Adaptation in the absence of chloramphenicol produced full protection against lethal concentrations of H_2O_2 and TSP. However, chloramphenicol addition during the adaptation period resulted in loss of protection against the lethal stress. This indicates that *de novo* protein synthesis during adaptation is necessary for protection. The five log decrease in viability observed in cells preadapted to H_2O_2 and simultaneously treated with chloramphenicol, prior to stress with the lethal concentration of H_2O_2, was not observed in TSP stressed cells. This may indicate that some of the systems providing protection against TSP are constitutive. This is perhaps not surprising. In *E. coli*, NhaA is both induced upon alkaline stress, but also expressed constitutively under non-stress conditions. In addition the activity of NhaA increases 10^3-fold upon a pH upshift from pH 7 to pH 8 (Hall *et al.*, 1995). The constitutively expressed ATPase and electron transport chain would aid in maintaining pH homeostasis in the absence of protein synthesis. However, whether these proteins are constitutively expressed in *C. jejuni* is unknown.

Survival of oxidative shock and TSP stress requires the production of proteins. Such stress responses can be examined by metabolic labelling of proteins synthesised during adaptation using [^{35}S]-methionine and analysis by 2D-PAGE (Section 6.9).

6.8 Effect of UV irradiation on the survival of *Campylobacter jejuni*

Exponential (12h) and stationary phase (24h) cells were examined for their relative resistance to UV irradiation and the effect of cold-shock for 2h at either 4 or 20°C on resistance to 10 ergs/mm^2 UV dose (Figure 6.13).

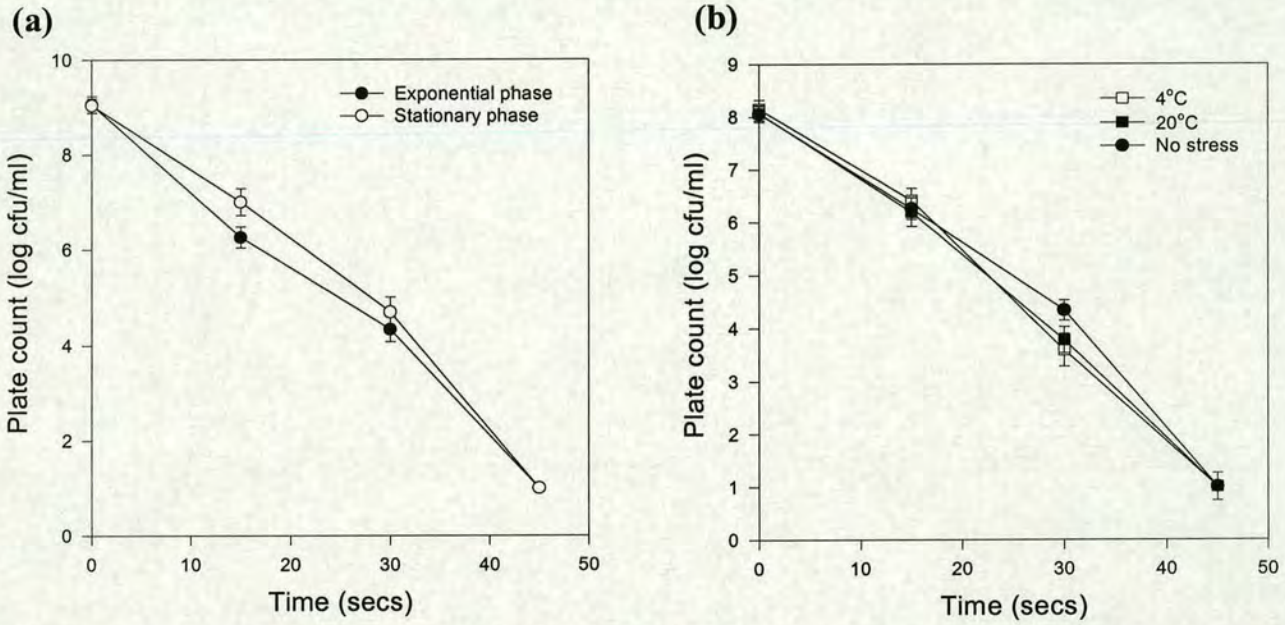


Figure 6.13: Effect of (a) growth phase, and (b) low temperature pre-incubation on *Campylobacter jejuni* 81116 survival to UV irradiation. Mean of two separate experiments

Incubation at either 4 or 20°C and entry into stationary phase did not affect the survival of *C. jejuni* to irradiation (Figure 6.14a,b). In all cases, no plateable cells were detected after 45 s irradiation at 10 ergs/mm^2. *C. jejuni* is known to be more sensitive to UV irradiation than other bacteria (Butler *et al.*, 1987), and it appears that UV irradiation would efficiently eliminate campylobacters regardless of the incubation temperature or physiological status of the cell, unlike the situation with H_2O_2 and TSP (Section 6.2). Despite the poor penetrative ability of UV irradiation, it may have practical use for the decontamination of *C. jejuni* from the surfaces of poultry products.

6.9 Effect of sublethal H_2O_2 and TSP stress on *de novo* protein synthesis in *Campylobacter jejuni* 81116

It is evident that adaptation of *C. jejuni* 81116 to sublethal concentrations of H_2O_2 or TSP requires *de novo* protein synthesis, perhaps indicating the production of specific stress proteins enabling survival (Section 6.6). *De novo* protein synthesis was investigated by 2D-PAGE analysis of radiolabelled proteins.

Over the duration of stress with 0.1 mM H_2O_2 (Figure 6.14) or 32 mM TSP (Figure 6.15), *C. jejuni* responds via the alteration of *de novo* protein synthesis of proteins, as indicated by 2D-PAGE. Some proteins are upshifted or specifically synthesised in response to the stress. It is these proteins that are of particular interest as they represent mechanisms by which *C. jejuni* survives oxidative shock or TSP stress. H_2O_2 induced proteins are examined in greater detail in the subsequent section (Section 6.10), initially examining the series of gels showing proteins synthesised in response to H_2O_2 over a 2h period (Figures 6.16.1-6.16.3), and then highlighting particular areas of interest in the form of montages (Figures 6.17.1-6.17.3). Similar analysis of the series of gels depicting proteins produced in response to TSP stress over a 45 min period is undertaken in Section 6.11.

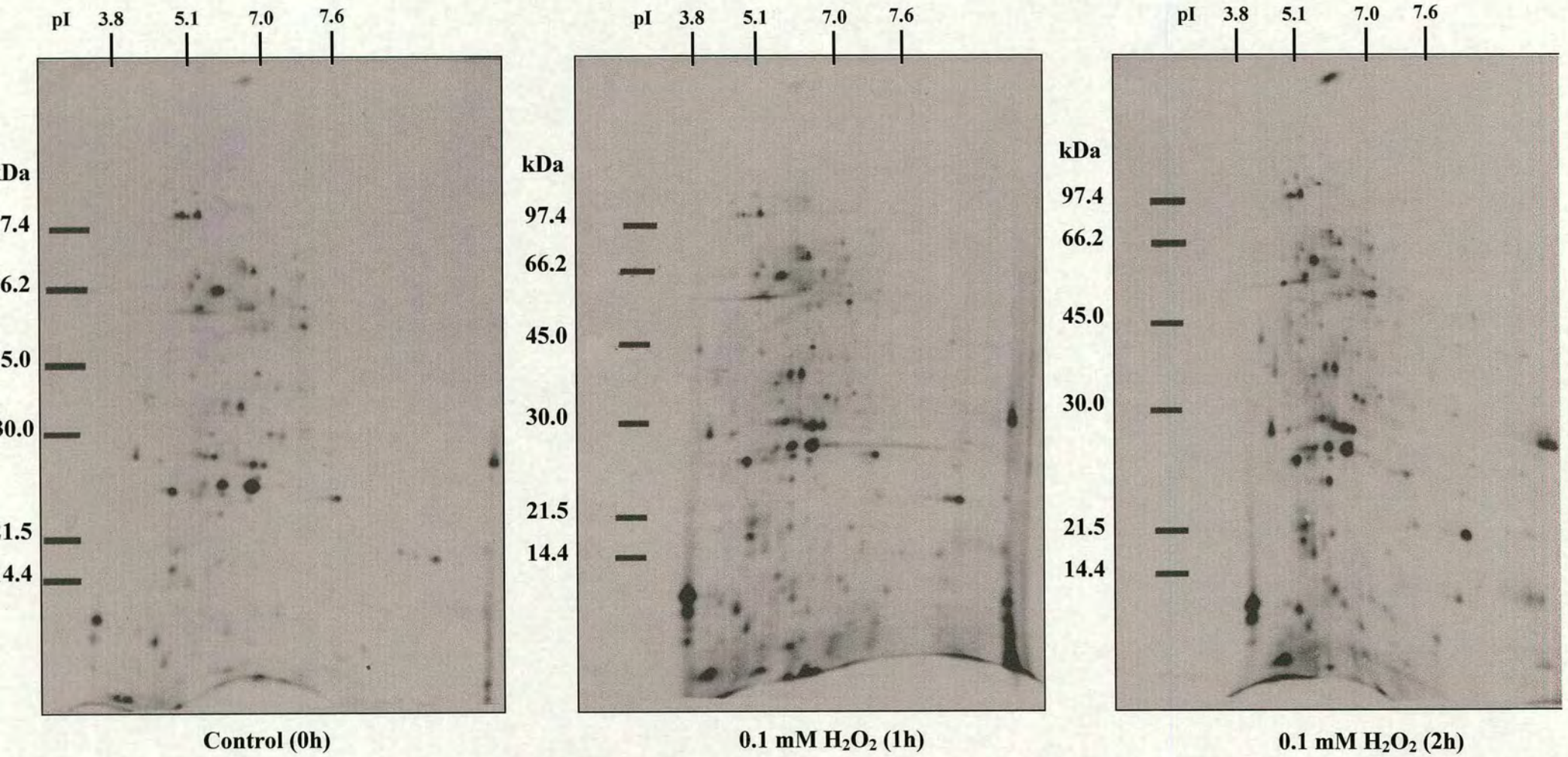


Figure 6.14: H_2O_2 induction of stress proteins in *Campylobacter jejuni* 81116. Cells were grown to ~10^8 cfu/ml in ABCD broth at 37°C under microaerobic atmosphere. Aliquots were incubated in the presence of 15 μCi/ml [^{35}S]-methionine for 15 min. Proteins (10μg) were then separated by 2D-PAGE, electroblotted and visualised by Enhanced Autoradiography

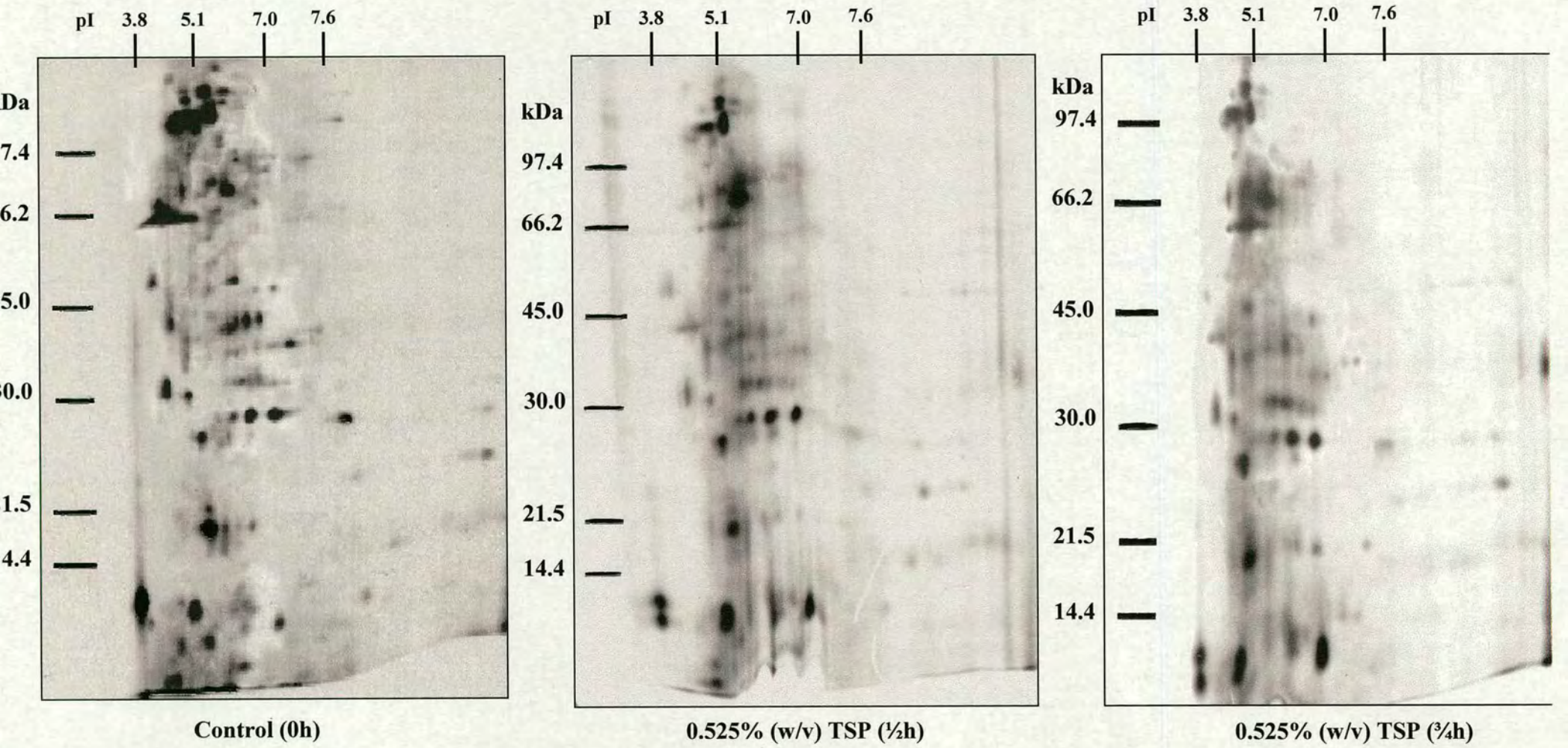


Figure 6.15: TSP induction of stress proteins in *Campylobacter jejuni* 81116. Cells were grown to ~10^8 cfu/ml in ABCD broth at 37°C under microaerobic atmosphere. Aliquots were incubated in the presence of 15 μCi/ml [^{35}S]-methionine for 15 min. Proteins (10μg) were then separated by 2D-PAGE, electroblotted and visualised by Enhanced Autoradiography

6.10 Effect of 0.1 mM H_2O_2 on the *de novo* protein synthesis of specific stress proteins in *Campylobacter jejuni* 81116

The gels were analysed using the 2D-Gel Analysis Software (Phoretix). Protein spots were identified that upon H_2O_2 stress increased from their basal levels present in the control (Figures 6.16.1 to 6.16.3). The software equalised the background intensities, and evaluated the molecular weight and isoelectric points of the proteins (Table 6.4). Proteins observed to have increased levels of synthesis compared to the control are designated as H, followed by the number designated to the spot on the control gel (e.g. H0). Proteins absent from the control but induced upon H_2O_2 stress are designated HS (hydrogen peroxide specific).

The proteins presented (H0-H111) in Table 6.4 and Figures 6.16-6.17 are synthesised pre-stress but in reduced amounts compared to post-stress. There are four different temporal kinetics for these proteins.

(a) Some proteins are synthesised in increased amounts over the 2h stress period (e.g. H24, H31, H42, H60, H74 and H77).

(b) Some proteins show markedly increased levels after 1h stress, before the levels are reduce 2h after stress application (e.g. H58, H92, H99, H104, H105, H106, H108 and H110).

(c) other proteins show increased levels over the first hour of stress, then maintain a similar level of synthesis into the second hour (e.g. H81, H96 and H111).

(d) Finally, some proteins show decreased levels of synthesis after 1h of stress before increasing again after 2h stress. (e.g. H0, H3 and H25); whether these proteins represent stress proteins induced after 2h stress or housekeeping proteins being restored to their pre-stress levels is presently unknown. Some proteins are specifically induced by H_2O_2 (HS120-125).

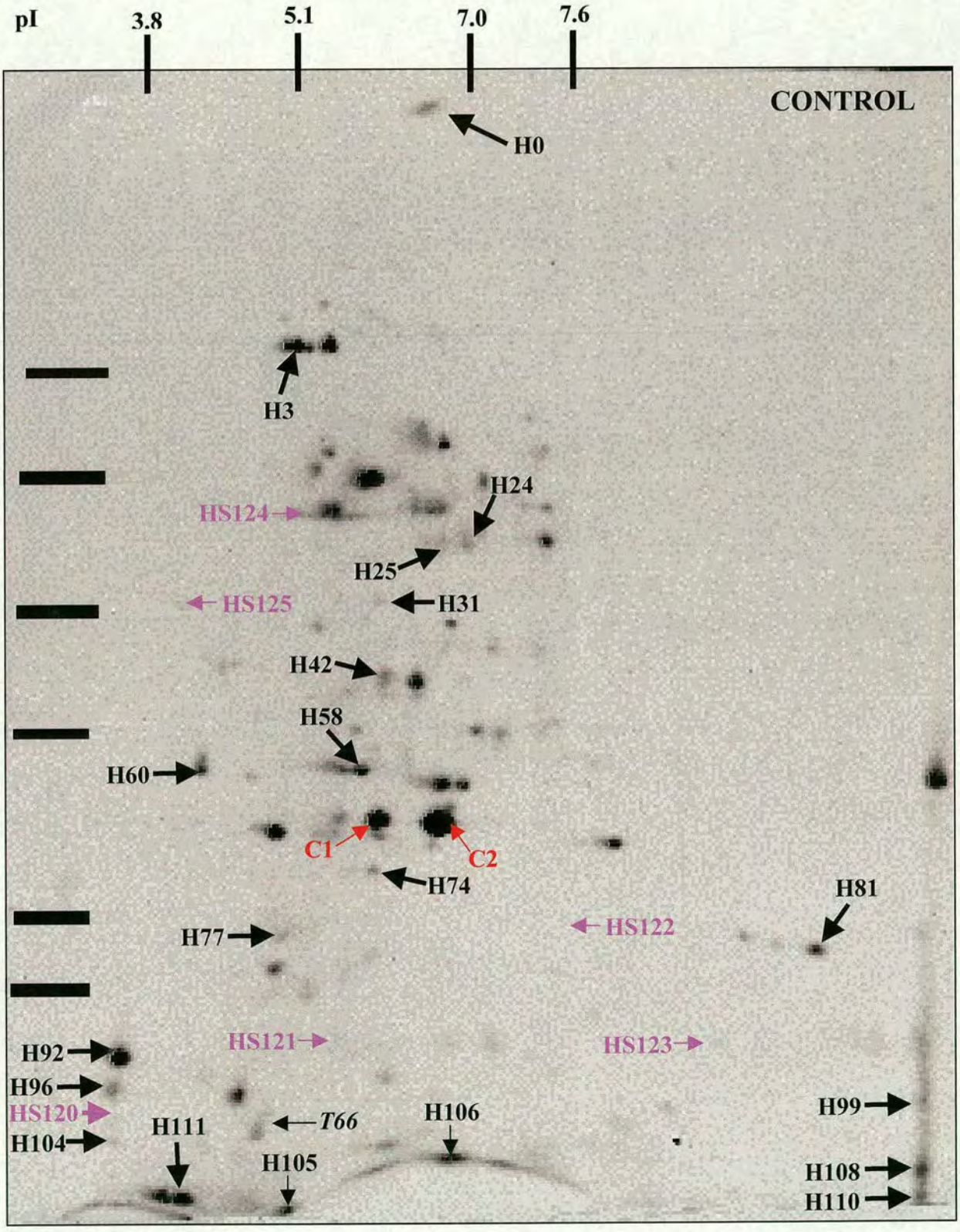


Figure 6.16.1: Proteins present in unstressed *Campylobacter jejuni* 81116 cells. Cells were incubated under microaerobic atmosphere at 37°C. Black arrows designate proteins, which are present in the control gel and whose synthesis is upshifted in response to H_2O_2. Purple arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2. Red arrows designate proteins induced at constant rates. T66 = protein induced in response to TSP stress

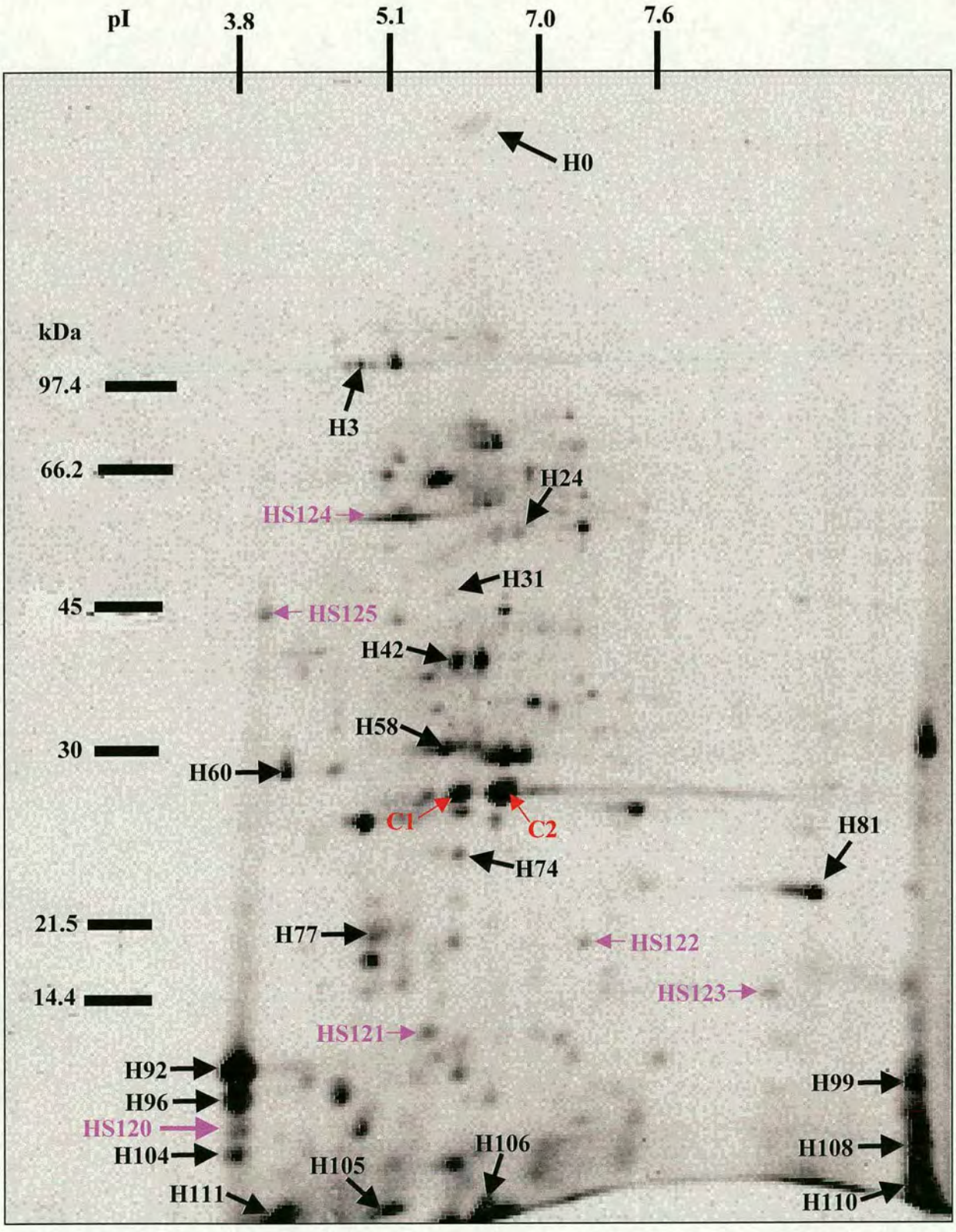


Figure 6.16.2: Proteins synthesised in response to 1h stress with 0.1 mM H_2O_2. Cells were incubated under microaerobic atmosphere at 37°C. Black arrows designate proteins that are present in the control gel and whose synthesis is upshifted in response to H_2O_2. Purple arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2. Red arrows indicate proteins induced at constant rates. T66 = protein induced in response to TSP stress

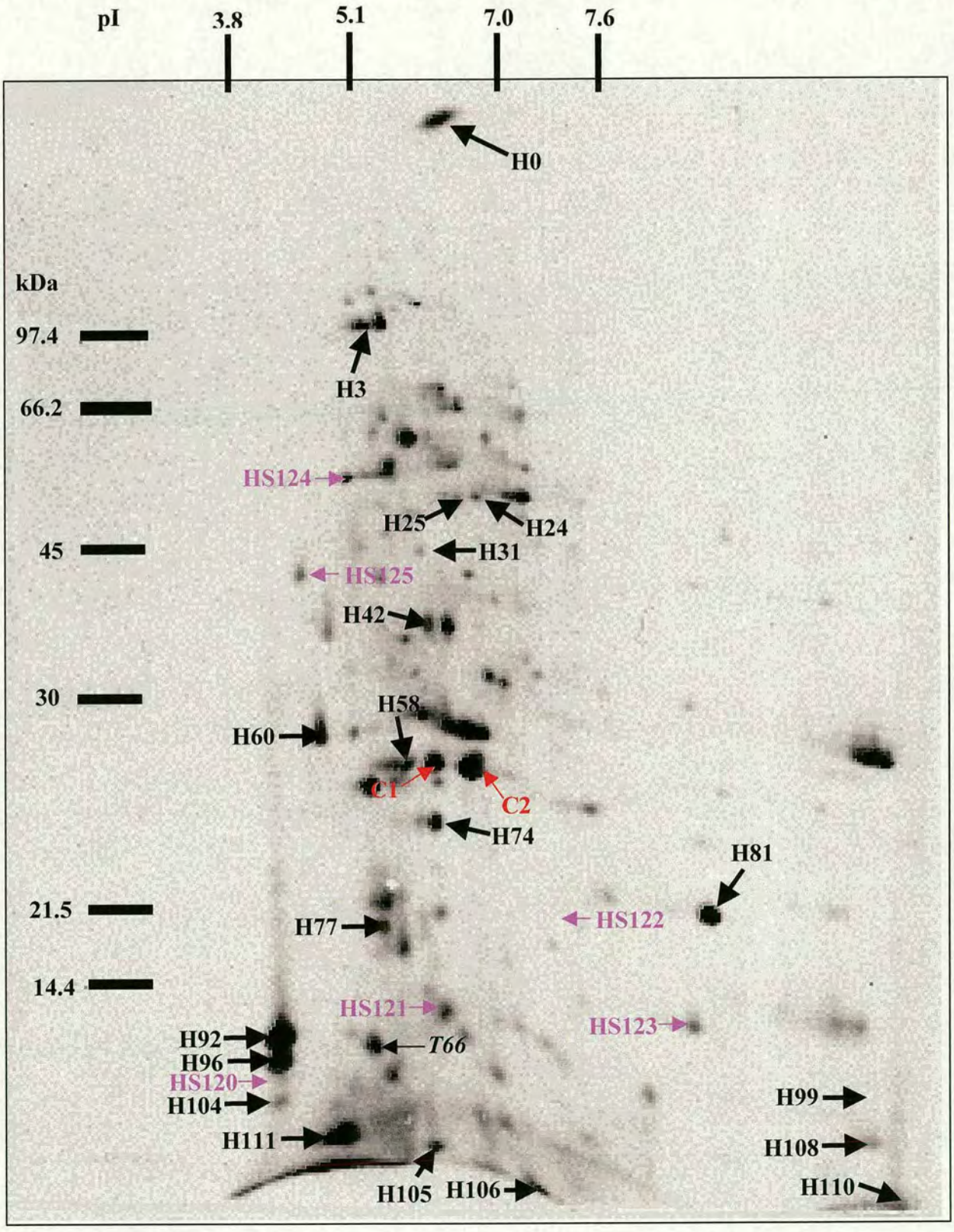


Figure 6.16.3: Proteins synthesised in response to 2h stress with 0.1 mM H_2O_2. Cells were incubated under microaerobic atmosphere at 37°C. Black arrows designate proteins that are present in the control gel and whose synthesis is upshifted in response to H_2O_2. Grey arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2. Red arrows indicate proteins induced at constant rates. T66 = protein induced in response to TSP stress

Protein	Mol. wt (kDa)	pI	Volume Control	Volume H_2O_2 (1h)	Volume H_2O_2 (2h)	Maximum induction (-fold)	Possible protein
H0	242.6	5.7	21475	10929	25098	2.3	-
H3	105.8	4.7	14526	7616	22109	2.9	-
H24	56.2	6.2	6904	8751	30804	4.5	KatA/GroEL
H25	55.9	6.1	9527	NP	19623	2.1	KatA/GroEL
H31	45.2	5.4	11741	11832	22271	1.9	HtpX
H42	36.1	5.4	14421	17294	25706	1.8	-
H58	27.6	5.2	15712	43212	23499	2.8	AhpC
H60	27.4	4.1	13447	20025	25726	1.9	AhpC
H74	21.1	5.3	12304	13854	19856	1.6	GrpE
H77	18.1	4.6	12509	21361	26667	2.1	Fur/Tpx
H81	17.5	9.0	15100	28683	27959	1.9	-
H92	14.2	3.6	25423	78947	59569	3.1	-
H96	13.4	3.6	14737	42758	37350	2.9	-
H99	13.0	10.1	13121	52308	NP	4.0	-
H104	12.3	3.6	11012	20320	14151	1.8	-
H105	12.3	5.4	12781	29677	NP	2.3	GroES
H106	12.0	5.9	35049	70998	NP	2.0	GroES
H108	11.7	10.1	18029	59574	NP	3.3	-
H110	11.3	10.3	16514	128151	NP	7.8	-
H111	11.3	4.0	20711	79988	68750	3.9	-
HS120	12.6	3.3	NP	1266	NP	-	-
HS121	14.0	5.2	NP	1505	2470	-	-
HS122	17.8	7.0	NP	501	NP	-	Fur/Tpx
HS123	14.1	8.7	NP	980	2412	-	-
HS124	62.1	4.5	NP	NP	3516	-	-
HS125	45.9	3.6	NP	1603	2333	-	-
C1							-
C2							-

Table 6.4: *De novo* protein synthesis profiles: Tabulated data of proteins present (H0-H111) or absent (HS120-H125) in the control gel (gel 0) and induced by H_2O_2 stress (0.1 mM) after 1h (gel 1) and 2h (gel 2). NP = not present

The volumes obtained are comparable between gels because the amount of protein loaded was equalised at 10 μg per gel. Furthermore some proteins are observed to be expressed at constant levels over the duration of the experiment (Table 6.4), acting as internal controls.

Four of the proteins are low molecular weight with very basic pI (H81, H99, H108 and H110). DNA is negatively charged; therefore DNA-binding proteins require an overall basic charge to enable binding. It is possible that these are DNA-binding proteins playing a role in the regulation of the peroxide stress response.

Three particular areas will be examined further and are highlighted as montages (Figures 6.17.1-6.17.3): (a) mol. wt <14 kDa, pI 3.8-5.4, (b) mol. wt 45-66 kDa, pI 5.4-7.6, and (c) mol. wt 21-30 kDa, pI 4.0-7.7

(a) Many of the low molecular weight acidic proteins are highly induced upon 1h stress with H_2O_2 (Figure 6.17.1). In the case of H96, synthesis is increased by 2.9-fold and this level is maintained for up to 2h. In contrast, H104 is induced 1.8-fold by 1h post-stress, before synthesis falls to pre-stress levels after 2h. It is possible that some proteins (e.g. H92, H96 and H104) represent the same protein, but differ in molecular weight due to modification or degradation.

(b) In contrast, the area of the gels between 66 and 45 kDa is less dynamic (Figure 6.17.2). However, two proteins in particular are induced as a result of peroxide stress at levels of 4.5- and 2.1-fold higher than in the control (H24 and H25 respectively). Interestingly, the calculated mol. wt of these proteins (H24 = 56.2 kDa; H25 = 55.9 kDa) are similar to the mol. wt of catalase (58.3 kDa) and GroEL (57.9 kDa) in *C. jejuni*. Both proteins are expressed in *C jejuni* in response to oxidative stress (Grant & Park, 1995 and Takata *et al.*, 1992).

(c) The area of the gels between 30 and 21 kDa (Figure 6.17.3) highlights the H_2O_2 induced expression of a number of proteins. In particular H58 and H60 have calculated mol. wts of 27.6 and 27.4 kDa respectively. The *C. jejuni* homologue of AhpC has a mol. wt of 26.0 kDa (Baillon *et al.*, 1999), hence one of these proteins may represent AhpC induction. H77 is induced 2.1-fold upon H_2O_2 stress, and has a calculated mol. wt similar to the iron uptake

regulatory protein (Fur) and thiol peroxidase (Tpx). Figure 6.17.3 highlights two proteins (designated 'C') which are synthesised at constant levels both before and after hydrogen peroxide stress.

These functional assignments to the protein spots are purely hypothetical, based on similarities between the calculated mol. wt obtained from the gels, and the known mol. wt of proteins involved in the survival of oxidative shock in *C. jejuni* (Table 7.4). Identification of induced proteins will be performed using 2D-PAGE analysis of total protein and nano-electrospray tandem mass spectrometry (Section 6.12).

In bacteria such as *E. coli*, *S. typhimurium* and *Bacteroides fragilis* there are separate responses for H_2O_2 and superoxide anions (Demple, 1993 and Rocha *et al.*, 1996). Whether the response to H_2O_2 in *C. jejuni* protects against superoxide anions or if there are separate responses is unknown. An adaptive response could be examined using paraquat as the source of superoxide anions.

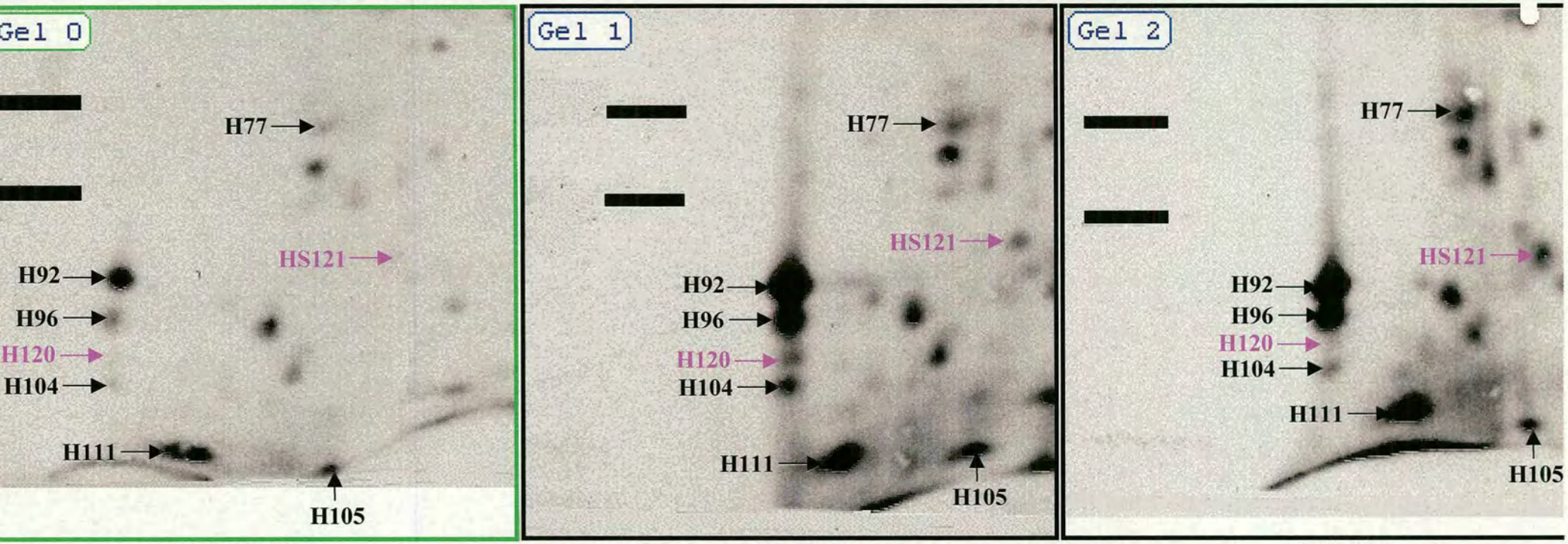


Figure 6.17.1: Montage of low mol. wt acidic proteins synthesised in response to 0.1 mM H_2O_2. Black arrows designate proteins that are present in the control gel and whose synthesis is upshifted in response to H_2O_2. Purple arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2

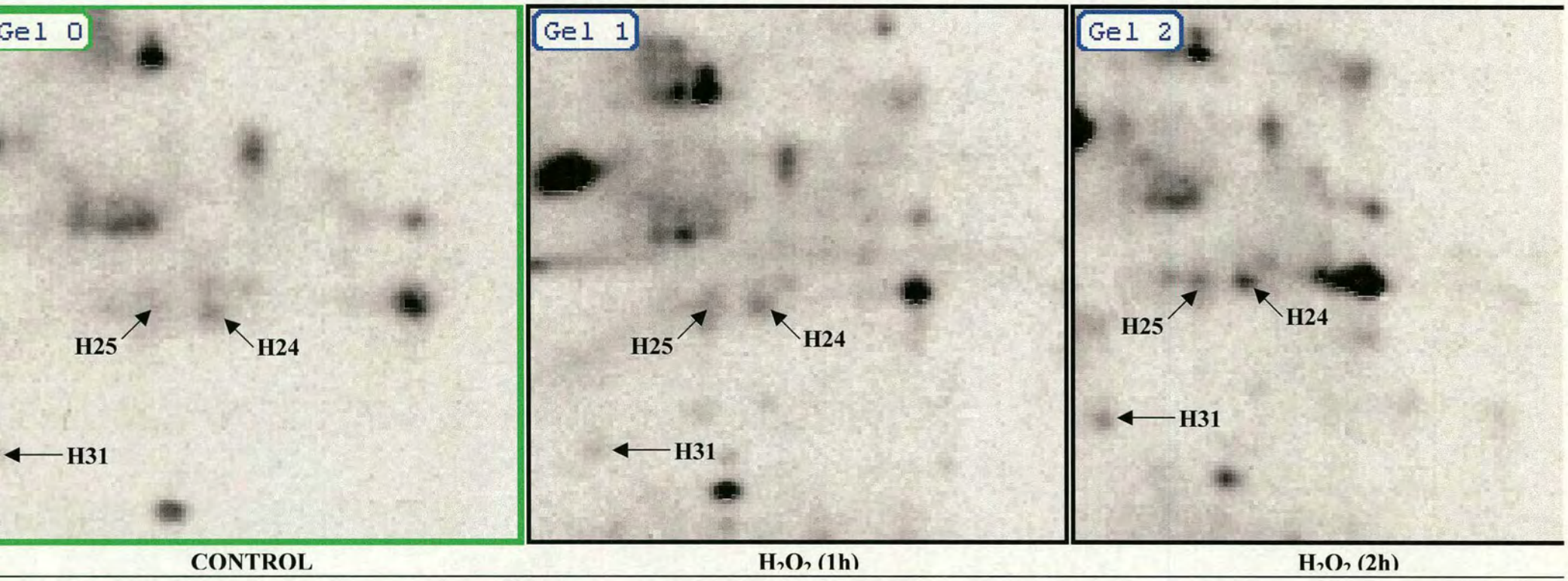
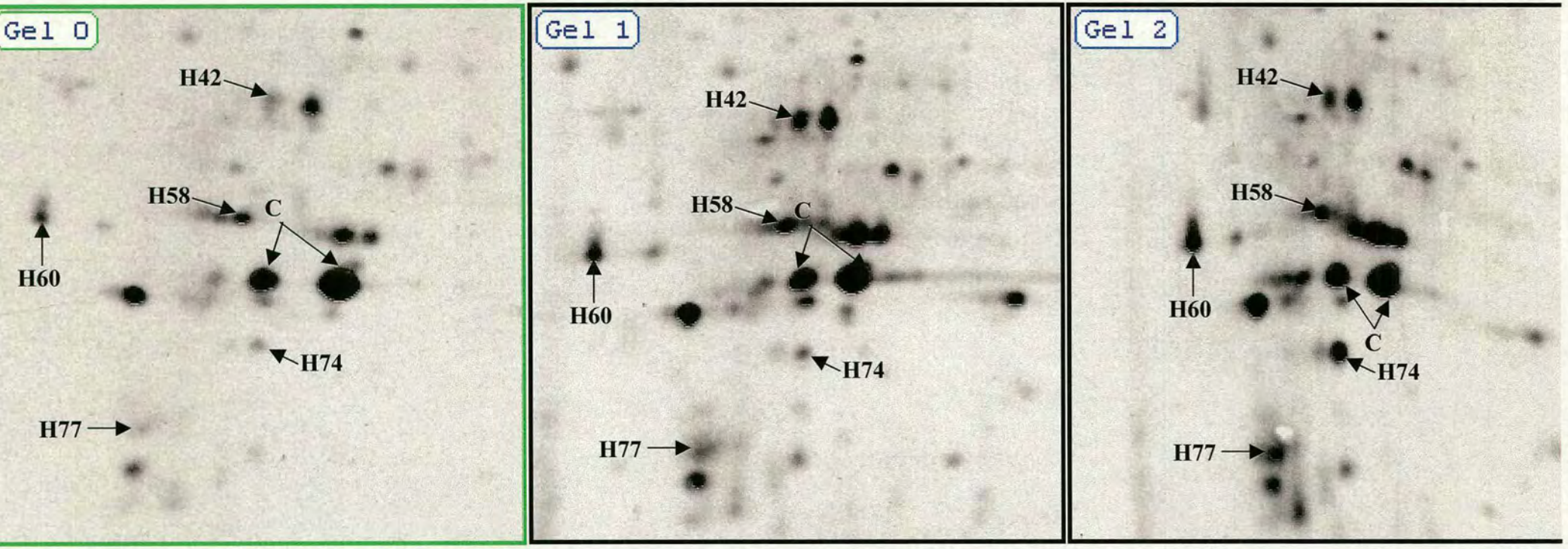


Figure 6.17.2: Montage of neutral proteins in the range 45-66 kDa synthesised in response to 0.1 mM H_2O_2. Black arrows designate proteins that are present in the control gel and whose synthesis is upshifted in response to H_2O_2. Purple arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2



CONTROL **H_2O_2 (1h)** **H_2O_2 (2h)**

Figure 6.17.3: Montage of neutral proteins in the range 21-30 kDa synthesised in response to 0.1 mM H_2O_2. Black arrows designate proteins that are present in the control gel and whose synthesis is upshifted in response to H_2O_2. Purple arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2. C = protein levels constant

6.11 Effect of TSP on the *de novo* protein synthesis of specific stress proteins in *Campylobacter jejuni* 81116

Proteins, whose cellular level of synthesis increased following TSP stress compared to untreated controls, were identified by using the Phoretix 2D-Analysis Software (Figures 6.18.1 to 6.18.3). The mol. wts and isoelectric points of the proteins were calculated by the software (Table 6.5). Proteins whose levels of synthesis increased compared to the control are designated as T, followed by the number designated to the spot on the control gel (e.g. T13). Proteins absent from the control but induced by TSP are designated as TS (trisodium phosphate specific).

Protein	Mol. wt (kDa)	pI	Volume control	Volume TSP (1h)	Volume TSP (2h)	Maximum induction (-fold)	Equivalent on H_2O_2 gels
T13	93.9	6.1	645	4030	NP	6.2	-
T22	67.1	5.7	2839	3417	7837	2.8	-
T65	14.3	6.8	1423	6256	7193	5.1	-
T66	13.9	5.3	7270	16238	12745	2.2	-
T69	14.1	8.5	3650	8601	9665	2.6	-
TS100	13.8	3.9	NP	4176	4412	-	H96
TS101	11.6	10.1	NP	907	5928	-	H110
TS102	37.3	9.9	NP	856	1778	-	-
TS103	24.6	8.1	NP	1529	1838	-	H81

Table 6.5: Tabulated data of *de novo* protein synthesis profiles of proteins induced upon TSP stress (32 mM) after 30 and 35 min. NP = not present

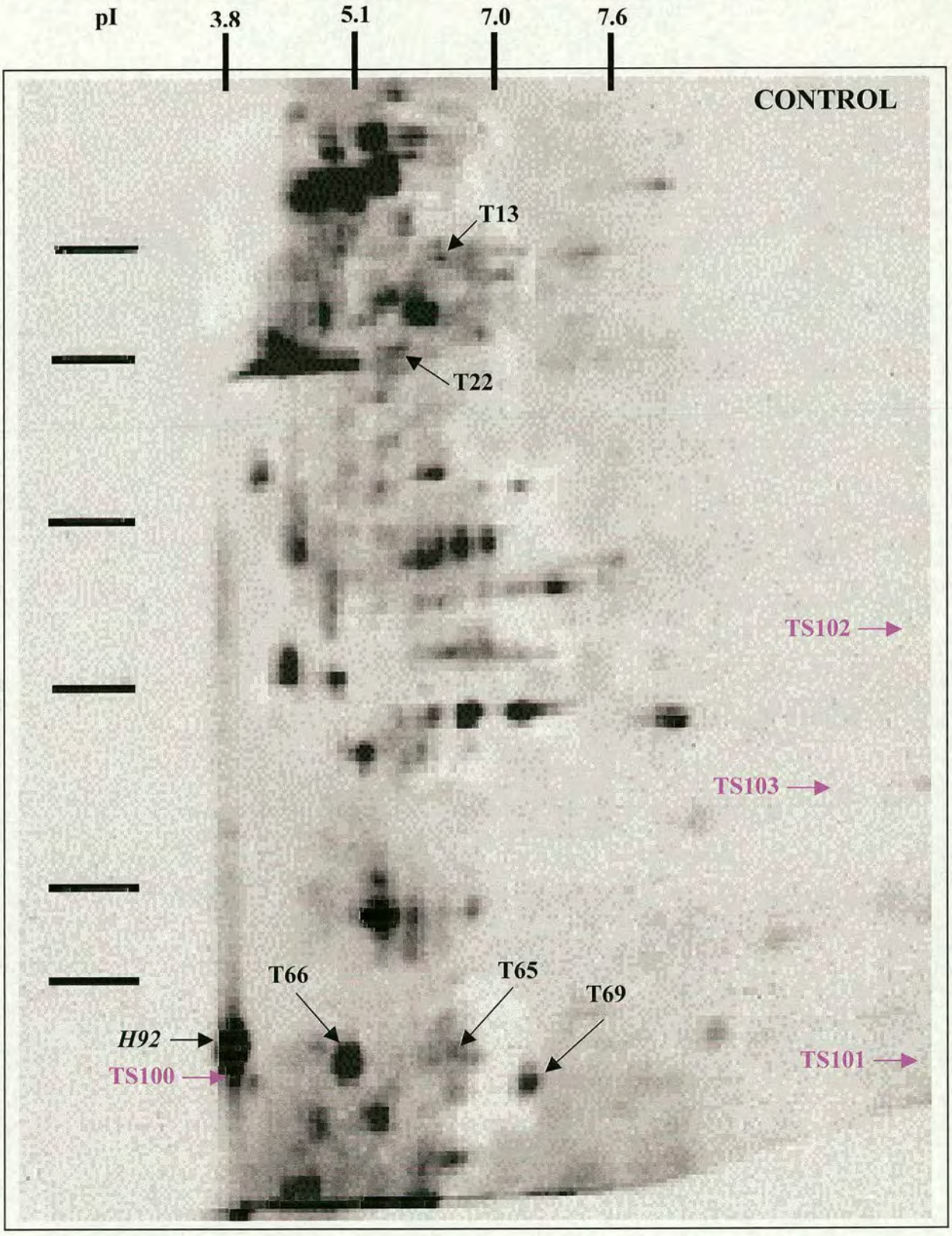


Figure 6.18.1: Proteins present in unstressed *Campylobacter jejuni* 81116 cells. Cells were incubated under microaerobic atmosphere at 37°C. Black arrows designate proteins, which are present in the control gel and whose synthesis is upshifted in response to TSP. Purple arrows designate proteins absent in the control gel but are synthesised in response to TSP. H92 = protein in response to H_2O_2 stress

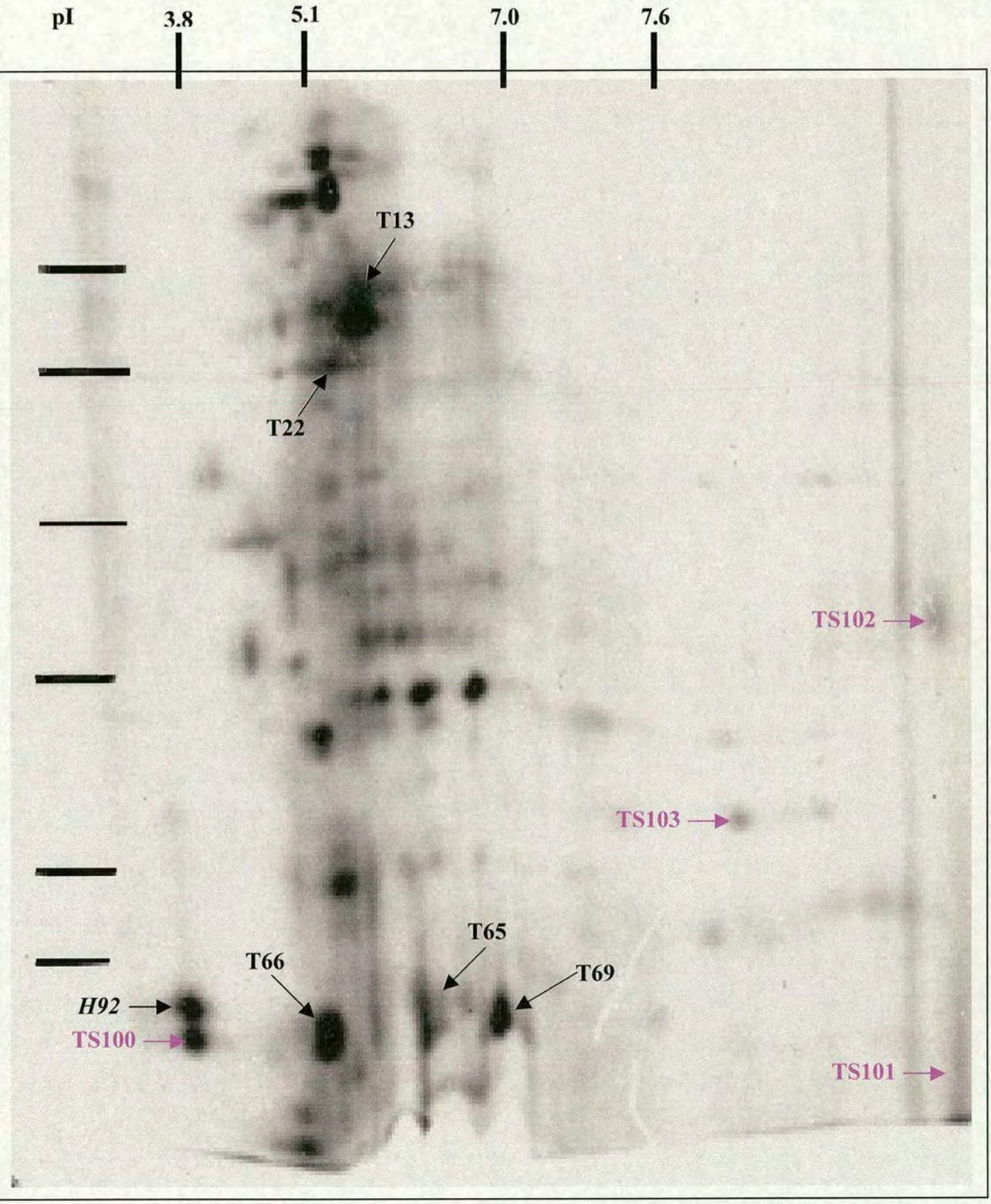


Figure 6.18.2: Proteins synthesised in response to stress for 30 min with 32 mM TSP. Cells were incubated under microaerobic atmosphere at 37°C. Black arrows designate proteins, which are present in the control gel and whose synthesis is upshifted in response to TSP. Purple arrows designate proteins absent in the control gel but are synthesised in response to TSP. H92 = protein induced in response to H_2O_2 stress

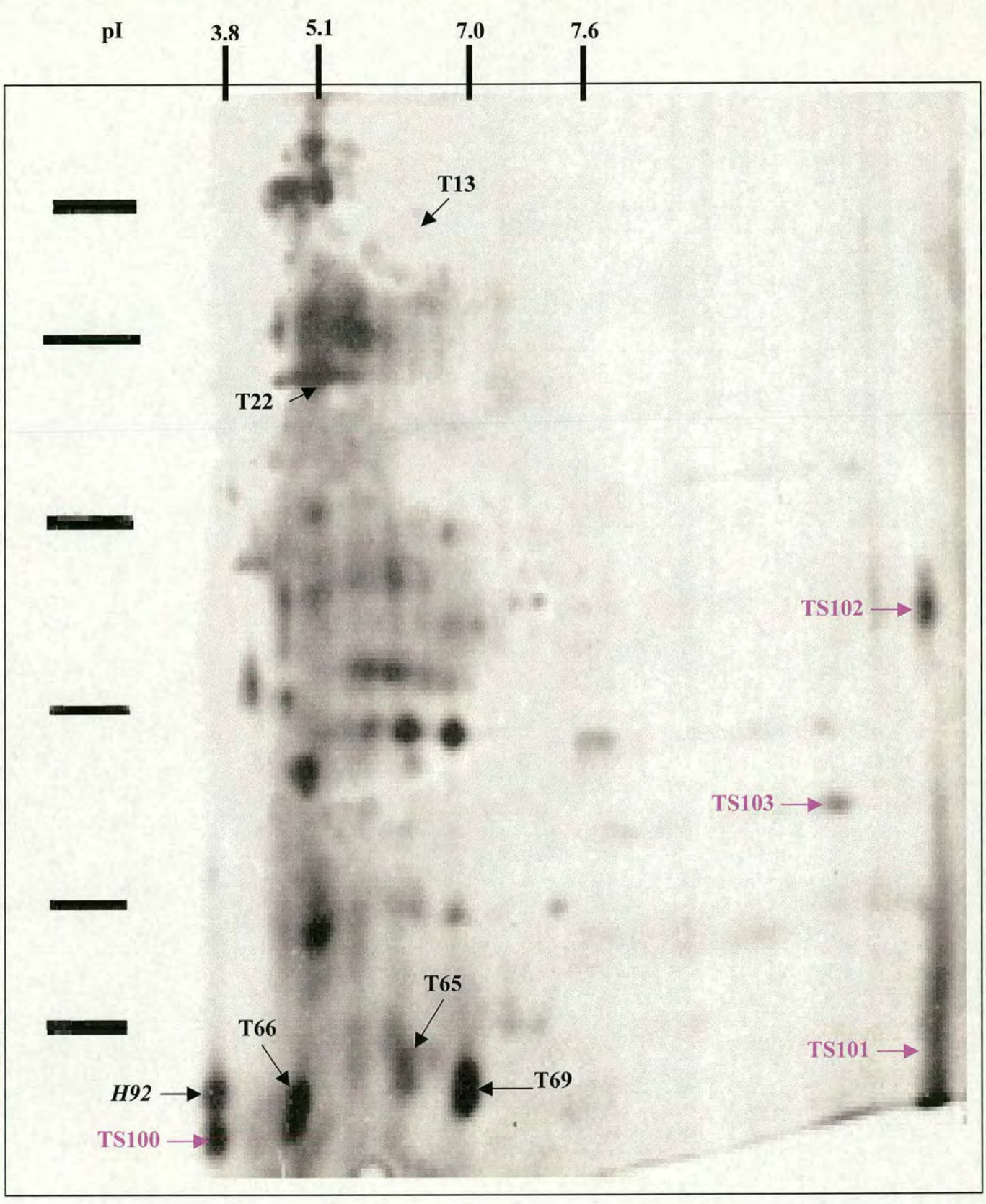


Figure 6.18.3: Proteins synthesised in response to stress for 45 min with 32 mM TSP. Cells incubated under microaerobic atmosphere at 37°C. Black arrows designate proteins, which are present in the control gel and whose synthesis is upshifted in response to TSP. Purple arrows designate proteins absent in the control gel but are synthesised in response to TSP. H92 = protein induced in response to H_2O_2 stress

A number of proteins (T22, T65 and T69) demonstrate increased synthesis over the stress period of 45 mins (Figures 6.18 and Table 6.5). One protein (T13) is synthesised in greater amounts after 30 mins, but synthesis is abolished after 45 mins. Another protein (T66) shows increased levels after 30 mins, before decreasing slightly by 45 mins. Some proteins (TS100-TS103) are not present in the control gel.

Fewer proteins were upshifted in response to TSP stress (at least 9 proteins), compared to hydrogen peroxide stress (at least 26 proteins). This may explain the phenomenon where chloramphenicol treatment during adaptation affected H_2O_2 resistance to a greater degree than TSP resistance (Figure 6.12). It is likely that *C. jejuni* resistance to the alkaline stress imposed by TSP solutions is partially independent of *de novo* protein synthesis. For example, the increased activity of Na^+/H^+ antiporters present in the membrane, or action of pH homeostatic mechanisms (ATPase or electron transport chain) may not be dependent on the induction of protein synthesis.

This thesis presents evidence for cross-protection between oxidative shock and TSP stress (alkaline). Adaptation with sublethal concentrations of H_2O_2 or TSP protected against lethal concentrations of the alternative stress (Figure 6.11). From the 2D-PAGE profiles (Figures 6.16 and 6.18), it is evident that proteins TS100/H96, TS101/H110 and TS103/H81, are identical and induced under both stresses. Similarly, H92 is present in TSP stressed cells, and T66 is present in cells stressed with H_2O_2 although the level of induction is low. It is interesting to note that in *Enterococcus faecalis*, alkalinity (pH 10.5) and 1.1 M NaCl provides cross-protection to H_2O_2 (Flahaut *et al.*, 1998). TSP appears to mediate its effects via alkaline pH, however, whether this provides cross-protection to stresses other than H_2O_2 is unknown.

6.12 Identification of proteins induced by H_2O_2 stress

In order to identify the H_2O_2 induced proteins observed by autoradiography (Figures 6.16.1-6.16.3), concentrated protein samples were separated by 2D-PAGE. Initially coomassie stained (GelCode Blue®) total protein profiles of *C. jejuni* 81116 cells subjected to 0.1 mM H_2O_2 for 1h (Figure 6.20.2) were compared to a control gel (no treatment; Figure 6.20.1). Proteins induced by H_2O_2 stress were identified (Figure 6.20.1-6.20.2 and Table 6.6), excised, digested with trypsin, and finally sequenced by nano-electrospray tandem mass spectrometry (Welmet protein sequencing facility). The sequence was then searched against known protein sequences in databases (SEQUEST c. V3).

Due to the higher background experienced on stained profile, it is more difficult to ascertain which proteins are upregulated in response to the stress. To circumvent this problem, proteins that can be visualised to be upregulated either on the full gels (Figures 6.19.1-6.19.2) or magnified areas of the gels (Figure 6.21) were chosen as the proteins of interest for identification by mass spectrometry. Other, low mol. wt proteins (<20 kDa) may be induced in response to H_2O_2, however, the gel background is too high to permit clear elucidation of this region. These low mol. wt proteins could be better separated using a tricine buffer system in conjunction with 2D-PAGE. Equalising the amounts of protein loaded on the control and stressed gels reduced experimental error. It should be noted that although 2D-PAGE separates proteins with greater resolution than 1D-PAGE, two proteins might exist in close proximity on the gel, possessing, similar mol. wts and isoelectric points. In such a situation, both proteins would be detected by mass spectrometry.

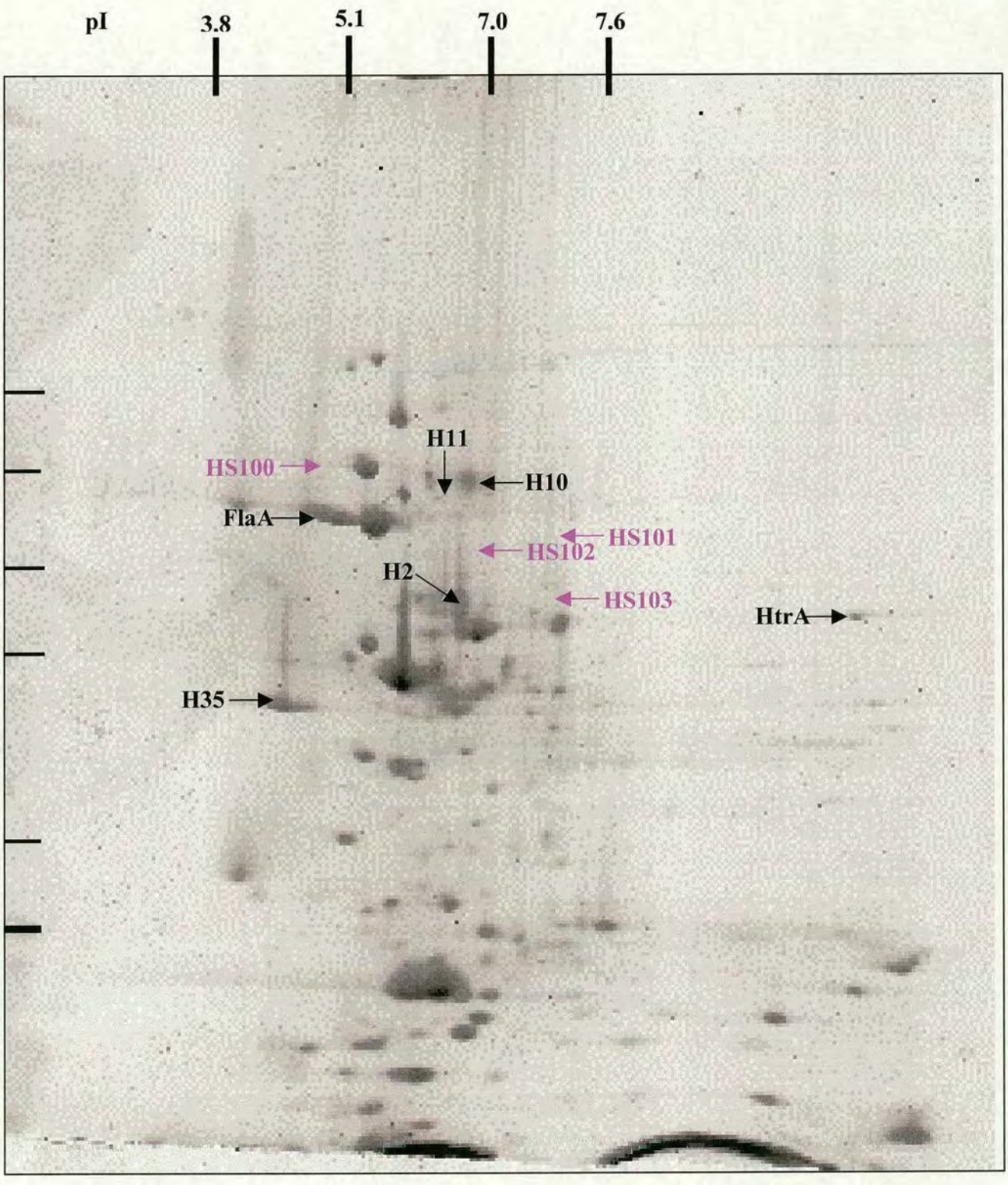


Figure 6.20.1: Proteins synthesised in unstressed *Campylobacter jejuni* 81116 cells. Cells were grown in ABCD broth to ~10^8 cfu/ml at 37°C under microaerobic atmosphere, before being stressed for the designated period of time. Proteins (10 mg) were then separated by 2D-PAGE. Proteins were visualised by staining with GelCode Blue® reagent. Black arrows designate proteins, which are present in the control gel and whose synthesis is upshifted in response to H_2O_2. Purple arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2

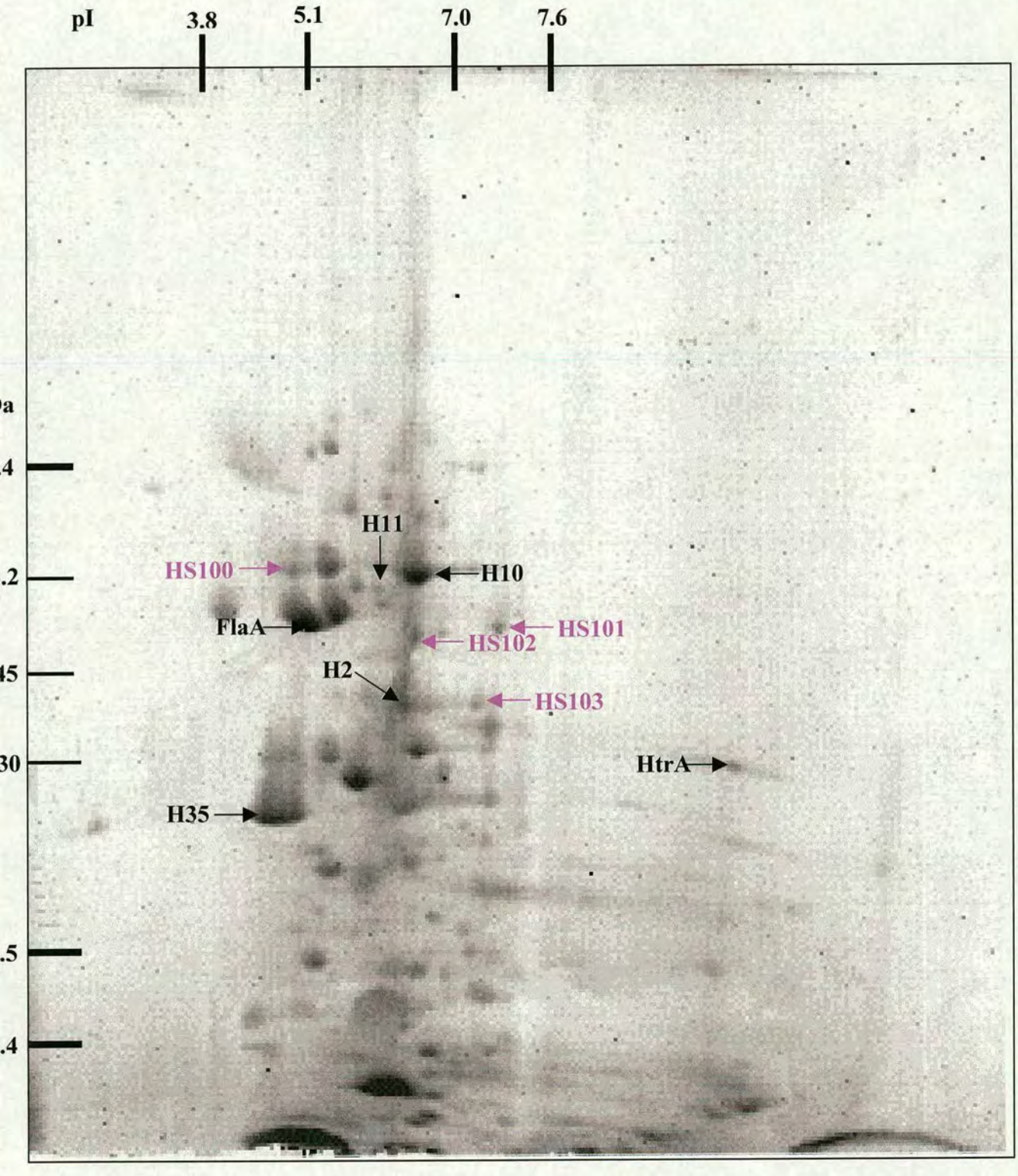


Figure 6.20.2: Proteins induced after 1h incubation with 0.1 mM H_2O_2. Cells were grown in ABCD broth to ~10^8 cfu/ml at 37°C under microaerobic atmosphere, before being stressed for the designated period of time. Proteins (10 mg) were then separated by 2D-PAGE. Proteins were visualised by staining with GelCode Blue® reagent. Black arrows designate proteins, which are present in the control gel and whose synthesis is upshifted in response to H_2O_2. Purple arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2

Protein	Mol. wt (kDa)	pI	Volume control	Volume H_2O_2 (1h)	Maximum induction (-fold)	Protein
H2	39.9	6.0	286	5347	18.7	-
H10	62.8	6.5	2829	7965	3.8	-
H11	58.4	5.8	349	819	2.5	-
H14	56.2	4.5	2069	11907	5.8	FlaA
H21	32.7	9.8	2328	3984	2.2	HtrA
H35	27.2	4.0	1814	19443	10.7	-
HS100	65.7	4.6	NP	1887	-	-
HS101	53.0	7.9	NP	3315	-	-
HS102	51.4	6.5	NP	2104	-	-
HS103	41.3	7.6	NP	429	-	-

Table 6.6: Total protein profiles: Tabulated data of proteins present (H2-H35) or absent (HS100-103) in the control gel and induced by H_2O_2 stress (0.1 mM) after 1h. NP = not present

A number of proteins were identified that fell into two different patterns of synthesis.

(a) those proteins present in the control gel, but significantly induced upon H_2O_2 stress (H2-H35).

(b) some proteins were absent from the control gel, but were synthesised in response to H_2O_2 stress (HS100-HS103).

Note that the profiles of the gels depicting *de novo* protein synthesis (Figures 6.16.1-6.16.3) differ from the total protein profiles (Figures 6.20.1-6.20.2). This is not unexpected, as radioactive labelling will detect protein induction more sensitively than total protein staining profiles. It is noteworthy, that as the cells were only stressed for 1h in this experiment, transiently expressed proteins and those induced after 2h could remain undetected. Nevertheless, at least three proteins are present in similar positions on the two profiles. On the total protein profiles, H2, H14 and H35, resemble, H42, HS124 and H60 respectively on the *de novo* protein synthesis profiles.

The majority of the proteins induced by H_2O_2, as visualised on the total protein profiles, are present in the region between 66.2 to 30.0 kDa. This region is magnified in Figure 6.22. A number of proteins are induced greater than three-fold in response to H_2O_2 stress (H2, H10, H14 and H35; Table 6.6). Others are absent in the control, but induced upon H_2O_2 stress (HS100-HS103). The identity of H14 was determined by nano-electrospray tandem mass spectrometry (Figures 6.21.1-6.21.4 and Table 6.6).

The tryptic digest of protein H14 was passed through the Thermoquest LCQ mass spectrometer revealing a succession of peaks representing peptide sequences (primary fragmentation; Figure 6.21.1).

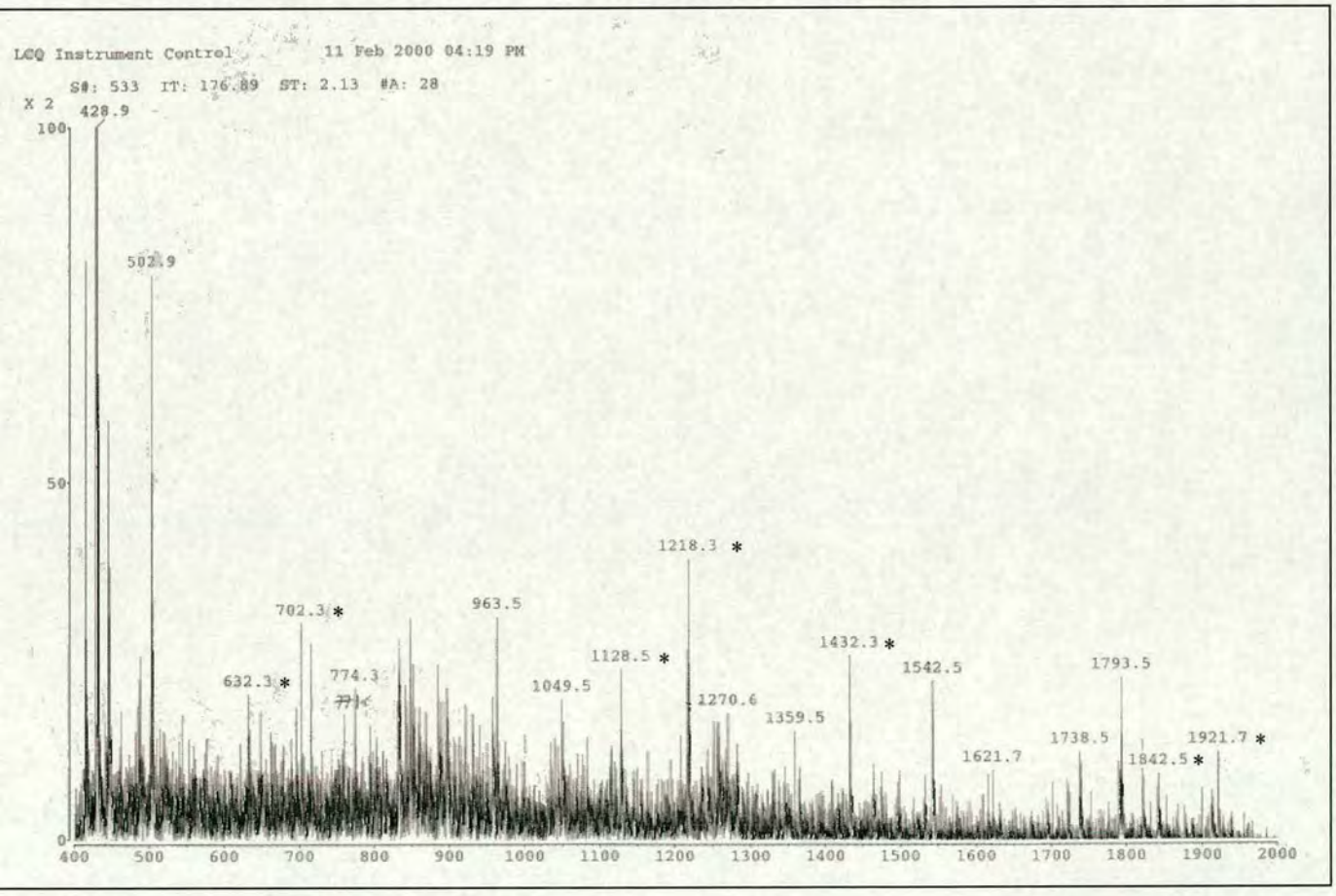


Figure 6.21.1: Primary fragmentation of the peptide profile from the tryptic digest of protein H14. The double charged ion at peak 1270.6 was chosen for secondary fragmentation Asterisks represent peptides present within protein H14

A double-charged peptide was then split further into peptides differing in mol. wt by an amino acid residue (secondary fragmentation; Figure 7.21.2). The elucidated amino acid sequence was then searched through a database (SEQUEST c.V 3), revealing the identity of the peptide sequence (1270.6) as the C-terminus of the Flagellin A protein of *C. jejuni*. Further checks to confirm the identity of the protein were performed by artificial tryptic digestion of the FlaA sequence using the Mascot software (http://www.matrixscience.com; Figure 6.21.3). The remaining single-charged ions from Figure 6.21.1, can then be checked for their appearance in the artificial digest. Seven peptide sequences were identified by this method, thus confirming the initial identification of protein H14 as Flagellin A. Figure 6.21.4 shows the full FlaA amino acid sequence, with the peptide regions observed by mass spectrometry, highlighted in underlined, bold typing. The predicted mol. wt of H14 was 56.2 kDa, which was close to the actual mol. wt of FlaA (58.9 kDa), providing further evidence linking protein H14 as flagellin A.

This is the first evidence suggesting environmental regulation of FlaA expression in *C. jejuni*. Previously it has been reported that growth atmosphere, alkaline pH (8.0-9.0) and growth temperature (higher at 42°C) regulate the expression of FlaB via the σ^{54} promoter (Alm *et al.*, 1993). However, why would FlaA be synthesised in response to H_2O_2 stress? It is known that *C. jejuni* possesses chemotactic responses to oxygen (Hazeleger *et al.*, 1998). Perhaps due to the microaerophilic nature of the organism, the addition of H_2O_2 (oxidative stress) triggers increased synthesis of FlaA to enable escape from the oxidative shock. Interestingly, the flagellum has been identified as one of the major virulence determinants of *C. jejuni*, allowing penetration of the intestinal mucosal epithelium (Ketley, 1997). Additionally, it has been reported that cells which have been subjected

to oxidative shock (increase dissolved O_2 tension from 4% to 120%) display increased infective capacity for Caco-2 cell lines (Harvey *et al.*, 1998). Although the mechanism is unknown, it may be that increased activity of the flagellum plays a role in this phenomenon, increasing the invasiveness of the stressed bacterium.

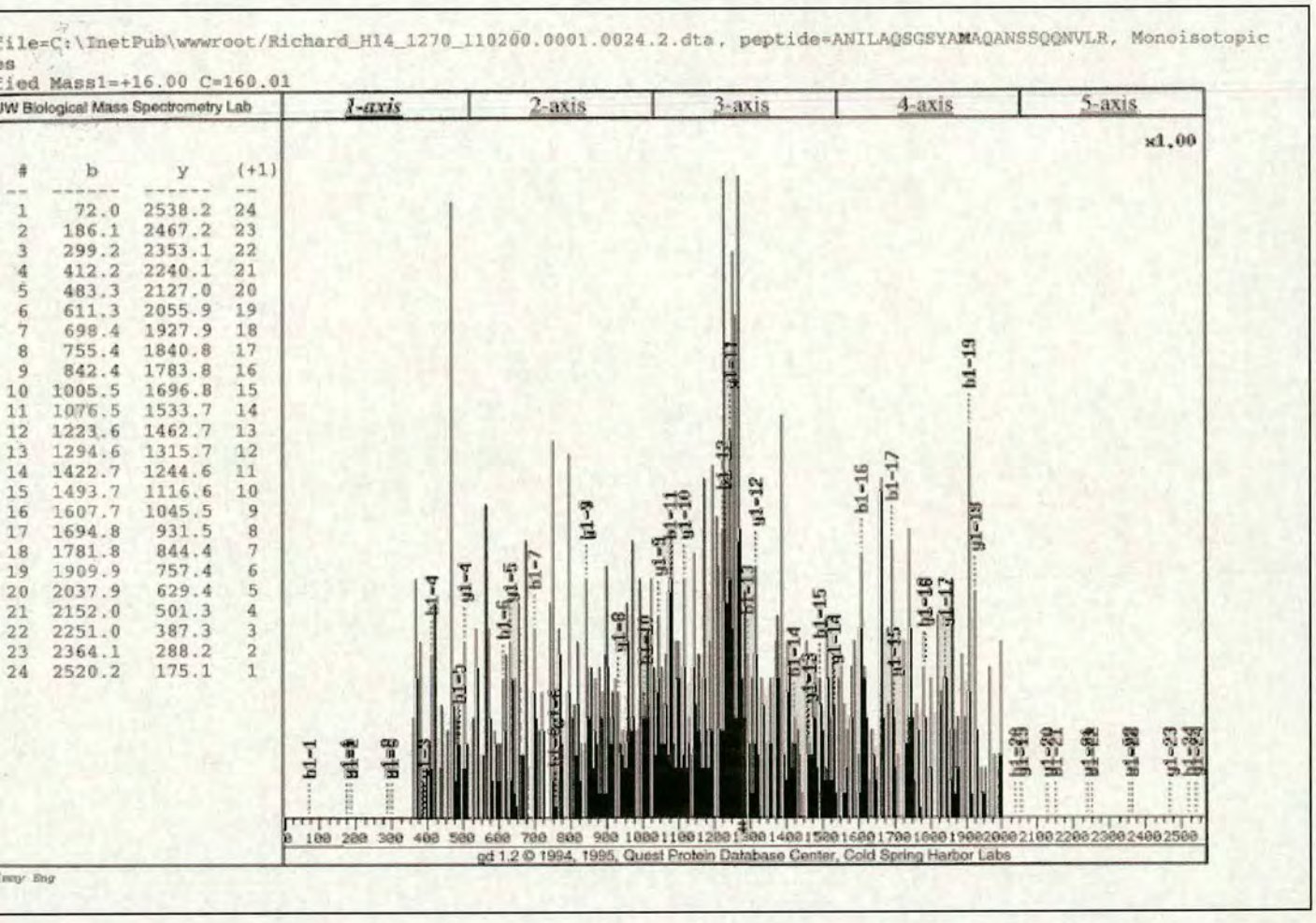
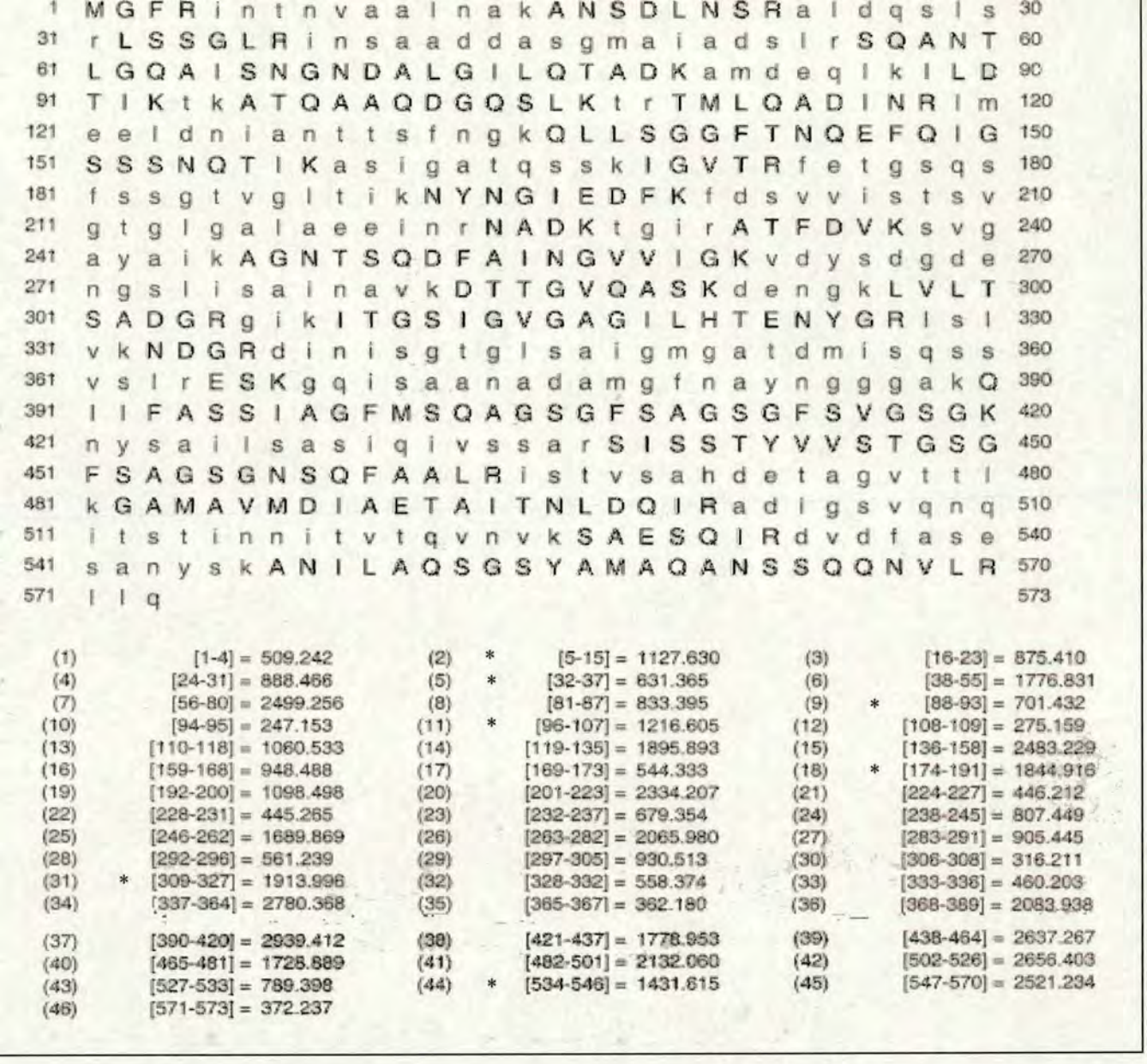


Figure 6.21.2: Secondary fragmentation of the 1270.6 double-charged peptide from protein H14 revealing the amino acid sequence (left hand side)



(1)		[1-4] = 509.242	(2)	*	[5-15] = 1127.630	(3)		[16-23] = 875.410
(4)		[24-31] = 888.466	(5)	*	[32-37] = 631.365	(6)		[38-55] = 1776.831
(7)		[56-80] = 2499.256	(8)		[81-87] = 833.395	(9)	*	[88-93] = 701.432
(10)		[94-95] = 247.153	(11)	*	[96-107] = 1216.605	(12)		[108-109] = 275.159
(13)		[110-118] = 1060.533	(14)		[119-135] = 1895.893	(15)		[136-158] = 2483.229
(16)		[159-168] = 948.488	(17)		[169-173] = 544.333	(18)	*	[174-191] = 1844.916
(19)		[192-200] = 1098.498	(20)		[201-223] = 2334.207	(21)		[224-227] = 446.212
(22)		[228-231] = 445.265	(23)		[232-237] = 679.354	(24)		[238-245] = 807.449
(25)		[246-262] = 1689.869	(26)		[263-282] = 2065.980	(27)		[283-291] = 905.445
(28)		[292-296] = 561.239	(29)		[297-305] = 930.513	(30)		[306-308] = 316.211
(31)	*	[309-327] = 1913.996	(32)		[328-332] = 558.374	(33)		[333-336] = 460.203
(34)		[337-364] = 2780.368	(35)		[365-367] = 362.180	(36)		[368-389] = 2083.938
(37)		[390-420] = 2939.412	(38)		[421-437] = 1778.953	(39)		[438-464] = 2637.267
(40)		[465-481] = 1728.889	(41)		[482-501] = 2132.060	(42)		[502-526] = 2656.403
(43)		[527-533] = 789.398	(44)	*	[534-546] = 1431.615	(45)		[547-570] = 2521.234
(46)		[571-573] = 372.237						

Figure 6.21.3: Predicted tryptic digest of the *Campylobacter jejuni* flagellin A (FlaA) protein using the Mascot software. Asterisks represent peptide sequences present within flagellin A

RINTNVAA LNAKANSDLN AKSLDASLSR **LSSGLR**INSA ADDASGMAIA DSLRSQANTL GQAISNGNDA LGILQTADKA
EQLKI**LDT** **IK**TK**ATQAAQ** **DGQSLK**TRTM LQADINKLME ELDNIANTTS FNGKQLLSGN FTNQEFQIGA SSNQTVKATI
TQSSKIGV TR**FETGAQSF** **TSGVVGLTIK** NYNGIEDFKF DNVVISTSVG TGLGALAEEI NKSADKTGVR ATYDVKTTGV
IKEGTTSQ DFAINGVTIG KIEYKDGDGN GSLISAINAV KDTTGVQASK DENGKLVLTS ADGRGIK**ITG** **DIGVGSGILA**
KENYGRLS LVKNDGRDIN ISGTNLSAIG MGTTDMISQS SVSLRESKGQ ISATNADAMG FNSYKGGGKF VFTQNVSSIS
MSAQGSGF SRGSGFSVGS GKNLSVGLSQ GIQIISSAAS MSNTYVVSAG SGFSSGSGNS QFAALKTTAA NTTDETAGVT
KGAMAVMD IAETAITNLD QIRADIGSIQ NQVTSTINNI TVTQVNVKAA ESQIR**DVDFA** **SESANYSKAN** **ILAQSGSYAM**
ANSSQQNV **LR**LLQ

Figure 6.21.4: Sequence of *Campylobacter jejuni* flagellin A protein (H14) showing regions of identified peptide homology identified by mass spectrometry in bold and underlined

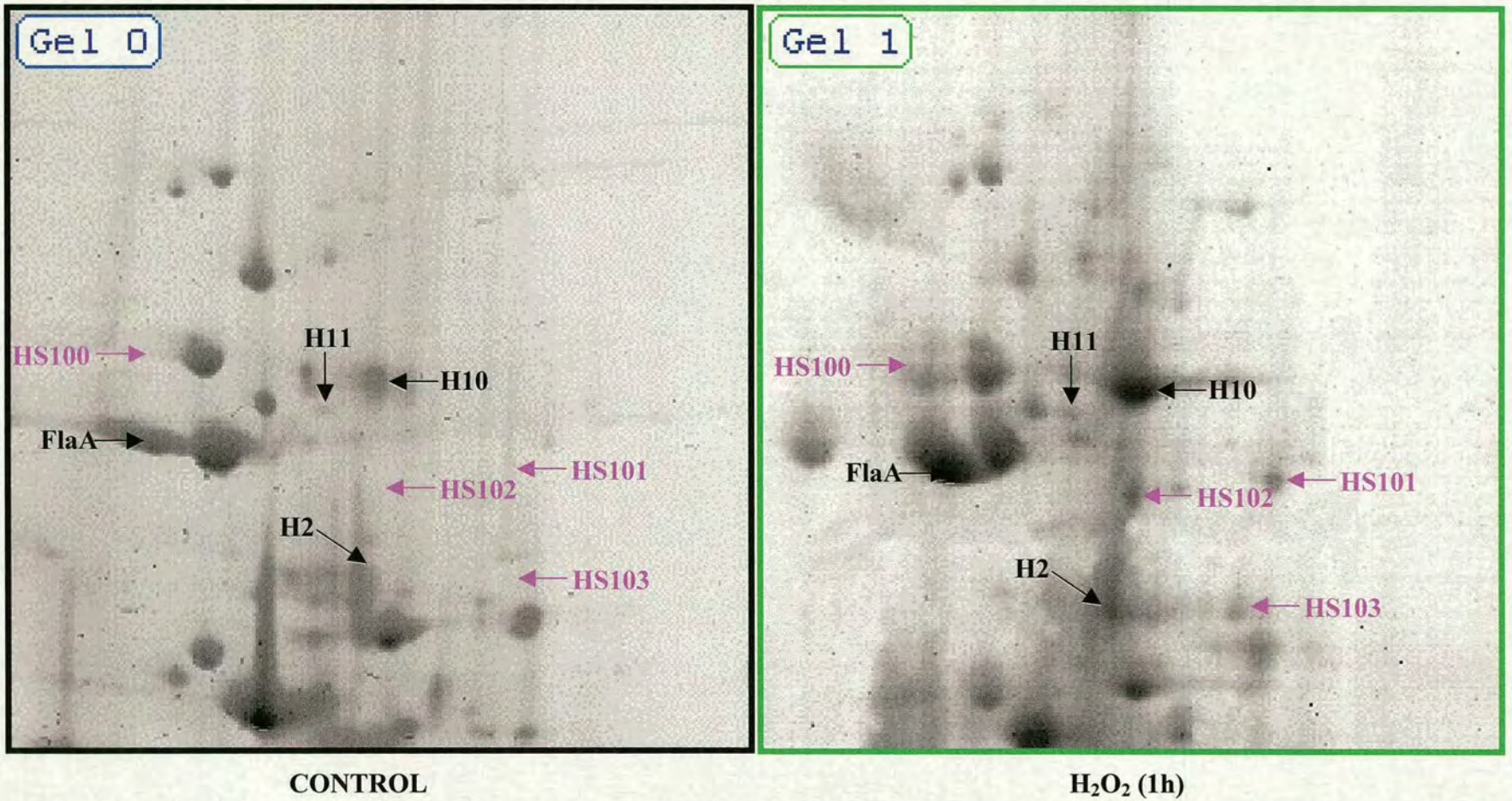


Figure 6.22: Montage highlighting the region of major H_2O_2 induced proteins in *Campylobacter jejuni* 81116. Black arrows designate proteins that are present in the control gel but upshifted in response to H_2O_2. Purple arrows designate proteins absent in the control gel but are synthesised in response to H_2O_2

Similarly, protein H21 was examined by mass spectrometry. Two double-charged ions were obtained, both corresponding to peptide sequences found within the serine protease Do (HtrA) of *C. jejuni* (Figure 6.23).

MKKIFLSLSL ASALFAASIN FNESTATANR VNPAAGNAVL SYHDSIKDAK KSVVNISTSK TITR**ANRPSP** **LDDFFNDPYF**
KQFFDFDFSQ RK**GKNDKEVV** **SSLGSGVIIS** **K**DGYIVTNNH VVDDADTITV NLPGSDIEYK AKLIGKDPKT DLAVIKIEAN
NLSAITFTNS DDLMEGDVVF ALGNPFGVGF SVTSGIISAL NKDNIGLNQY ENFIQTDASI NPGNSGGALV DSRGYLVGIN
SAILSRGGGN NGIGFAIPSN MVKDIAKKLI EKGKIDRGFL GVTILALQGD TKKAYKNQEG ALITDVQKGS SADEAGLKRG
DLVTKVNDKV IKSPIDLKNY IGTLEIGQKI SLSYERDGEN KQASFILKGE KENPKGVQSD LIDGLSLRNL DPRLKDRLQI
PKDVNGVLVD SVKEKSKGKN SGFQEGDIII GVGQSEIKNL KDLEQALKQV NKKEFTKVWV YRNGFATLLV LK

Figure 6.23: Sequence of *Campylobacter jejuni* serine protease Do (HtrA; H21) showing regions of identified peptide homology identified by mass spectrometry in bold and underlined

In *E. coli*, serine protease Do (HtrA) is induced during heat-shock (Yura & Nakahigashi, 1999). This represents the first indication of the protease being induced in response to oxidative shock, certainly in *C. jejuni*. The function of proteases during heat-shock is to degrade misfolded, aggregated and denatured proteins. It is probable that similar problems befall proteins when subjected to oxidative shock, hence the need for degradative proteases such as HtrA to be induced as part of the adaptive response. The mol. wt of HtrA is predicted to be 51.0 kDa from the genome. However, the mol. wt of protein H21 (shown to be HtrA) is only 32.7 kDa. This may be due to differences in migration on 2D-PAGE, or alternatively, perhaps the right hand side of the gels in Figures 6.20.1 and 6.20.2 are distorted slightly in favour of the lower mol. wt.

6.13 Summary

The bactericidal action of TSP was demonstrated to be primarily due to the alkaline pH of the solutions (Figures 6.1-6.3). Sensitivity to alkaline pH was dependent on the presence of Na^+ or K^+ ions (Figure 6.3), possibly implicating the actions of Na^+/H^+ and K^+/H^+ antiporters in the resistance.

Incubation at low temperature (4°C) protected *C. jejuni* 81116 cells from the lethal effects of TSP, H_2O_2, acid (pH 3.8), NaCl and NaOCl experienced at 20-37°C (Figures 6.6 and 6.7).

Adaptation to sublethal concentrations of H_2O_2 and TSP protected against lethal doses of up to 5 mM H_2O_2 and 61 mM TSP respectively (Figure 6.12). Stationary phase cells did not exhibit increased resistance to lethal concentrations of both stresses compared to exponential phase cells. However, both growth phases exhibited adaptation (Figure 6.10). Cross-protection was observed between the two stresses (Figure 6.11).

Adaptation was dependent upon *de novo* protein synthesis (Figure 6.9). Two-dimensional PAGE analysis of proteins synthesised under H_2O_2 and TSP stress revealed the synthesis of specific stress proteins (Figures 6.16 and 6.18), including some which were induced under both stresses, perhaps providing a basis for cross-protection. Flagellin A was identified as a H_2O_2 induced protein, providing the first evidence of environmental regulation of the major flagellin of *C. jejuni*. Additionally, serine protease Do (HtrA) was induced by H_2O_2 challenge.

Section 7

Analysis of the sequenced *Campylobacter jejuni* genome

7.0 Results and discussion: Analysis of the sequenced *Campylobacter jejuni* NCTC 11168 genome

The genome of *C. jejuni* NCTC 11168 has recently been sequenced by the Sanger Centre (http://www.sanger.ac.uk/Projects/C_jejuni/). Initial analysis of the putative coding regions (Section 9.0) has revealed insights into the capabilities of *C. jejuni* when confronted by unfavourable conditions. This will allow a closer link to be drawn between genomics and proteomics in future. This will permit techniques including 2D-PAGE, mass spectrometry, SELEX (systematic evolution of ligands by exponential enrichment), analysis of knockout mutants and DNA microarray technology to be combined, permitting elucidation of the regulatory circuits involved in survival and infection.

The data displayed in Section 9.0 and used in this section was personally generated during the period of study utilising search engines and the genome sequence found at the aforementioned website. The amino acid sequences of proteins synthesised in response to stress in other bacteria e.g. thiol peroxidase (Tpx), were compared against the decoded amino acid sequence of the *C. jejuni* genome using the Sanger Centre search engine. The stresses have been subdivided, and also include analysis of putative regulatory elements present within the genome. Subsequent, independent analysis of the sequence has been performed by the Sanger Centre, and a complete list of the proteins can be found at the website. Both the Sanger Centres lists and mine were similar, however, differences are highlighted in the tables. It should be noted that often what is absent in the genome reveals just as much as proteins that are present. Proteins are given along with their Cj coding number denoted by the Sanger Centre.

7.1 Regulatory proteins

Specific regulatory proteins regulate the expression of all genes, including those involved in stress responses. A number of known and putative regulatory components have been identified in the *C. jejuni* genome (Table 7.1).

Protein	Cj number	Mr (kDa)	pI	Comments
RpoA	1595	37.7	-	DNA-directed RNA polymerase alpha chain
RpoB	0478	155.9	-	DNA-directed RNA polymerase beta chain
RpoC	0479	168.8	-	DNA-directed RNA polymerase beta' chain
RpoD	1001	72.6	-	Normal metabolic genes
RpoF	0061c	28.4	-	Flagella and chemotaxis genes
RpoN	0670	48.7	-	Nitrogen regulated genes
RpoZ *	1273c	12.3	-	DNA-directed RNA polymerase omega chain
Fnr	0466	23.2	-	Fumarate and nitrate reduction regulator
??	1000	34.2	-	LysR-like
CsrA	1103	0.84	-	Carbon storage regulator
??	0643	46.7	-	Response regulator
DniR †	0645	42.8	-	Nitrite reductase regulator
Fur	0400	18.1	-	Iron regulator
PerR	0322	15.9	-	Peroxide regulator
KdgR †	0480c	29.3	-	Transcriptional repressor
HspR	1230	14.6	-	Heat shock protein transcriptional regulator
HrcA	0757	31.8	-	Heat-inducible transcriptional repressor
ArsR †	1561	0.69	-	Arsenical resistance operon repressor
RacR	1261	25.4	-	Temperature associated colonisation
RacS	1262	47.7	-	Temperature associated colonisation
GppA	0353c	55.2	-	Guanosine pentaphosphate phosphohydrolase
??	1222c	45.2	-	Histidine kinase
??	1223c	25.6	-	Response regulator
??	1226c	48.5	-	Histidine kinase
OmpR	1227c	25.5	-	Response regulator
NtrC †	1024c	49.2	-	Response regulator
??	1042c	35.0	-	AraC-like regulatory protein
CopR †	1491c	25.8	-	Transcriptional activator
??	1608	34.7	-	Response regulator
MerR †	1563c	17.0	-	Mercury resistance transcriptional regulator

Table 7.1: Regulatory proteins detected within the *Campylobacter jejuni* NCTC 11168 genome.
?? = unknown protein, † = proteins found by both me and the Sanger Centre, but only given a name based on homology by me, * = Proteins found by the Sanger Centre only

The core RNA polymerase enzyme is $\alpha_2\beta\beta'$ (RpoABC). Various sigma factors when bound determine the transcription of genes (Booth, 1999). In *E. coli*, these include, RpoD (normal metabolic genes), RpoF (flagella and chemotaxis genes), RpoN (nitrogen regulated genes), RpoH (heat-shock genes), RpoE (extreme heat-shock genes), and RpoS (growth phase regulated genes). *C. jejuni* does not possess RpoE, RpoH or RpoS, hence it would appear that *C. jejuni* has different regulatory systems for heat-shock and stationary phase responses, than those observed in many Gram-negative bacteria. This is perhaps because, like *H. pylori*, the genome is one-third the size of *E. coli*, and only possesses three (or four) sigma factors (RpoD, F, N and Z) and five two-component signal transduction pathways, possibly due to its specialised niche. *C. jejuni* has few two-component regulatory systems. Recently one has been characterised (RacR-RacS) involved in temperature associated adaptive responses, and is important in colonisation, regulating the heat-shock protein, DnaJ (Brás *et al.*, 1999). It is possible that one of the sigma factors (RpoD, F or N) functions as a regulator of stress responses. The putative *rpoZ* gene may represent an alternative sigma factor in *C. jejuni* for regulating gene expression. In *Streptomyces*, *rpoZ* encodes a sporulation specific sigma factor (Nováková *et al.*, 1998).

In *E. coli*, CsrA is a regulatory protein that post-transcriptionally controls bacterial gene expression. Numerous stationary phase genes are repressed and exponential phase metabolic pathways activated. Examples include, glycogen synthesis, gluconeogenesis, glycolysis, motility and adherence (Romeo, 1998). A CsrA homologue is present in the genome of *C. jejuni*. The role of this homologue may be similar to its function in *E. coli*.

7.2 Starvation/ stationary phase stress proteins

Many organisms adapt to entry into stationary phase and starvation with the involvement of gross morphological, biochemical and physiological changes (Section 1.2.6). In many Gram-negative bacteria this is mediated by a specific sigma factor, RpoS. Although RpoS regulates the expression of a number of stationary phase induced proteins (Section 1.2.6), other proteins are regulated by alternative mechanisms, for example ppGpp or cAMP-CRP. Some known stationary phase genes have been detected in the *C. jejuni* genome (Table 7.2).

Protein	Cj number	Mr (kDa)	pI	Comments
CstA	0917c	77.1	-	Carbon starvation protein A
CsrA	1103	8.4	-	Carbon storage regulator
SurE	0293	30.6	-	Stationary phase survival
Cfa	1183c	45.7	-	Cyclopropyl fatty acid synthetase
CydAB	0081-0082	58.2/ 41.9	-	Cytochrome BD oxidase
TopA	1686c	79.1	-	Topoisomerase I
CbpA	1229	33.3	-	Co-chaperone-curved DNA binding protein
SpoT	1272c	83.8	-	Stringent response: ppGpp synthesis
GppA	0353c	55.2	-	Stringent response: pppGpp synthesis

Table 7.2: Stationary or starvation proteins present in *Campylobacter jejuni* NCTC 11168

C. jejuni does not possess an RpoS homologue, therefore regulation of such responses is not via this mechanism. However, SpoT and GppA are present, therefore regulation via ppGpp or pppGpp is a possibility. Also, a protein showing homology to the catabolite repressor protein (CRP) has been observed in the genome. CstA promotes peptide utilisation when the carbon source is scarce. Cyclopropyl fatty acid synthetase catalyses the formation of cyclopropyl fatty acid derivatives, increasing membrane fluidity during starvation. CydAB encodes a cytochrome *d* oxidase, with high affinity for oxygen. It is expressed during stationary phase in *E. coli* when

oxygen becomes limiting, replacing the cytochrome *o* oxidase system (Siegele *et al.*, 1996). SurE is an L-isoaspartyl O-methyltransferase, essential for stationary phase survival in *E. coli*, supposedly repairing damaged protein residues (Li *et al.*, 1997). TopA encodes topoisomerase I which alters supercoiling in DNA. This can affect many cellular processes including DNA replication, transcription, recombination and DNA repair. Many promoters are sensitive to the superhelical density of the DNA template, and a number of environmental stresses result in a change in DNA supercoiling e.g. osmotic stress and nutrient depletion (Qi *et al.*, 1997). CbpA is an analogue of DnaJ, but is not induced during heat-shock, instead responding to entry into stationary phase or phosphate starvation (Yamashino *et al.*, 1994).

C. jejuni possesses the *rel*A-independent mechanism of generating ppGpp, via SpoT, a component of the stringent response to amino acid starvation. In addition, GppA synthesises pppGpp which also plays a role in the regulation of the response to amino acid starvation (Mukherjee *et al.*, 1998).

7.3 Oxidative stress defence

C. jejuni is a microaerophile, and therefore possesses an obligate respiratory metabolism dependent on oxygen. Components of the electron transport chain produce endogenous reactive oxygen intermediates (ROIs) such as H_2O_2 and superoxide anions, causing damage to many cellular components, including DNA, proteins and lipids. Adaptation to oxidative shock results in the upregulation of numerous proteins (Section 1.2.5 and Section 6.0). *C. jejuni* possesses some of these proteins involved in defence against reactive oxygen species (Table 7.3).

(i) Regulation of the oxidative stress response

C. jejuni does not possess OxyR, SoxRS, or ArcAB which are known to regulate oxidative stress responses in Gram-negative bacteria. Gram-positive bacteria possess the PerR protein that regulates the peroxide stress response. *C. jejuni* possesses a PerR homologue, which represses AhpC and KatA expression (van Vliet *et al.*, 1999). In addition evidence from the genome suggests the presence of a Fnr homologue (fumarate and nitrate regulator).

Protein	Cj number	Mr (kDa)	pI	Comments
KatA	1385	58.3	-	Catalase
SodB	0169c	24.8	-	Iron-dependent superoxide dismutase
AhpC	0334	26	-	Alkyl hydrogenperoxidase C subunit
RecA	1673c	37.1	-	Recombination protein
Tpx	0779	18.4	-	Thiol peroxidase
TrxB	0146c	33.1	-	Thioredoxin reductase
TrxB	0559	33.7	-	Thioredoxin reductase
Fnr	0466	23.2	-	Fumarate and nitrate reduction regulator
DnaENXQ	0718, 0002, 1157, 0452	137, 41, 58, 29	-	DNA polymerase III alpha, beta, gamma and epsilon chains
GroES	1220	9.5	5.2	Heat-shock protein
GroEL	1221	57.9	4.9	Heat-shock protein
PerR	0322	15.9	-	Peroxide stress regulator
Cytochrome c_{551} peroxidase	0020c	34.3	-	Removal of hydrogen peroxide
Cytochrome c peroxidase	0358	36.8	-	Removal of hydrogen peroxide

Table 7.3: Proteins involved in defence against oxidative shock in *Campylobacter jejuni* NCTC 11168

(ii) Detoxification

In *E. coli*, three SODs are present, two are cytoplasmic (Fe-SOD, Mn-SOD), whilst one is periplasmic (Cu/Zn-SOD), as well as two catalases, KatE (stationary phase induced) and KatG (H_2O_2 induced). Only one SOD, requiring iron as a co-factor, is present in *C. jejuni*, as well as one catalase, homologous to KatG. The reasons for possessing Fe-SOD rather than Mn-SOD or Cu/Zn-SOD is unclear, however, it makes sense to encode a H_2O_2 induced KatG homologue rather than the

stationary phase induced (and RpoS regulated) KatE protein due to the sensitivity of *C. jejuni* to oxidative stress.

Only AhpC is present, with no AhpF homologue being detected. However, in *S. typhimurium*, AhpC can work in conjunction with NADH oxidase, in the absence of AhpF (Marais *et al.*, 1998). *C. jejuni* possesses two cytochrome c peroxidases which may play a role in sequestering hydrogen peroxide. Tpx detoxifies hydroperoxides aided by glutathione or thioredoxin (reduced by TrxB) in a thiol dependent manner (Cha & Kim, 1999), whilst DNA polymerase and RecA are involved in the repair of DNA damage (Farr & Kagoma, 1991).

It is known that *C. jejuni* possesses an adaptive response to H_2O_2 (Section 6.0), as indicated by 2D-PAGE, with the upshift of approximately 24 proteins, including flagellin A (FlaA) and a serine protease (HtrA). It is likely that many of the unidentified proteins correspond to proteins present in Table 7.3. Synthesis of FlaA may aid in relocation of *C. jejuni* to a more favourable environment, whilst HtrA probably degrades misfolded, aggregated and damaged proteins as a result of oxidative shock.

7.4 Heat-shock proteins

C. jejuni needs to respond to temperature upshift, as a result of heating during the cooking of poultry products and also perhaps during fever induced due to infection in the body. Most bacteria possess proteins involved in the heat-shock response, involved in the correct folding of nascent proteins. DnaJ, DnaK and GrpE stabilise and prevent aggregation of newly synthesised and unfolded proteins, whilst GroES and GroEL organise folding of the proteins (Booth, 1999). As evidenced from searching the genome, *C. jejuni* has many heat-shock proteins (Table 7.4).

(i) Regulation of the heat-shock response

C. jejuni does not possess a RpoH homologue, hence regulation of the heat-shock response is not via this mechanism, unlike in *E. coli* and *S. typhimurium*. Intriguingly, in most bacteria, the heat shock response is negatively regulated, rather than positively regulated as in the case of RpoH. Regulatory systems present in other bacteria include HrcA, HflB (FtsH), sigma B, HspR and CtsR. No homologues for sigma B or CtsR were found.

Protein	Cj number	Mr (kDa)	pI	Comments
DnaJ	1260c	41.5	-	Stabilisation of unfolded proteins
DnaK	0759	67.3	4.8	Stabilisation of unfolded proteins
DksA	0125c	13.7	-	DnaK suppressor protein
GrpE	0758	20.3	-	Stabilisation of unfolded proteins
GroES	1220	9.5	5.2	Chaperone: folds proteins
GroEL	1221	57.9	4.9	Chaperone: folds proteins
HtpG	0518	69.7	-	Chaperone: folds proteins
HtpX †	0723c	45.3	-	Zinc-dependent metallo protease
HrcA	0757	31.8	-	Heat-inducible transcriptional repressor
HspR	1230	14.6	-	Heat shock protein transcriptional regulator
FtsH/HflB	1005c	61.3	-	Zinc metallopeptidase
FtsH/HflB	1116c	71.0	-	Zinc metallopeptidase
ClpA	1108	80.4	-	ATP-dependent Clp protease ATP-binding subunit
ClpP	0192c	21.7	-	ATP-dependent Clp protease proteolytic subunit
ClpX	0275	47.0	-	ATP-dependent Clp protease ATP-binding subunit
ClpB	509c	95.5	-	ATP-dependent Clp protease subunit
Lon	1073c	90.1	-	ATP-dependent protease La
HtrA	1228c	51.0	-	ATP-dependent serine protease Do
HslU	0662c	49.8	-	ATP-dependent protease
HslV	0663c	19.6	-	ATP-dependent protease

Table 7.4: Heat-shock proteins detected within the genome of *Campylobacter jejuni* NCTC 11168.
† = proteins found by both me and the Sanger Centre, but only given a name based on homology by me

C. jejuni possesses homologues of HspR and HrcA, known negative regulators of the heat-shock response in other organisms. Interestingly, *C. jejuni* possesses two homologues of the FtsH protein which functions as a protease, degrading RpoH in *E. coli* (Minder *et al.*, 2000). Whether it functions in a similar

manner with other heat-shock regulatory components (e.g. HrcA or HspR) is unknown. *Streptomyces coelicolor* possesses HspR, a transcriptional regulator, which binds to three inverted repeats in the promoter region of the *dna*K operon. In *H. pylori* HspR has been shown to negatively regulate the expression of *gro*ESL, *hrc*A-*grp*E-*dna*K and *cbp*A-*hsp*R-*orf* operons. These operons are transcribed by the vegetative sigma factor, RpoD (Spohn & Scariato, 1999). A similar system probably exists in *C. jejuni* where the arrangements of the aforementioned operons are conserved.

C. jejuni also possesses a HrcA homologue which is a heat-inducible transcriptional repressor. HrcA is modulated by the GroESL chaperonin system in *B. subtilis*. Under non-heat shock conditions HrcA-GroESL binds to DNA elements (CIRCE) present within the promoter region of heat-shock genes, repressing transcription. Upon heat-shock, repression is relieved as HrcA becomes inactive due to the involvement of GroESL in refolding denatured proteins, hence transcription of the downstream gene occurs (Minder *et al.*, 2000). A CIRCE sequence was found upstream of the *hrc*A-*grp*E-*dna*K operon of *C. jejuni* (Theis *et al.*, 1999c).

The induction of proteins in response to a temperature upshift of 37°C to 46°C was observed via 2D-PAGE (Konkel *et al.*, 1998). HspR, HrcA, RacRS and FtsH provide mechanisms by which this response may be regulated.

(ii) Molecular chaperones and proteases

C. jejuni has many of the known protein chaperones which aid in refolding denatured proteins. These include GroES, GroEL, DnaK, DnaJ, GrpE and HtpG. Proteases such as ClpAP, ClpB, ClpX, Lon, HtrA, and HslUV act by degrading denatured or misfolded proteins. The function of DksA is unknown.

7.5 Cold-shock proteins

C. jejuni is exposed to low temperatures on refrigerated poultry produce and upon entry into the environment such as water systems. Many mesophilic bacteria have a cold-shock response (Section 1.2.3), however, this is absent in *C. jejuni* (Section 5.13). Nevertheless, *C. jejuni* possesses a number of proteins present in the cold-shock response of other organisms (Table 7.5).

Protein	Cj number	Mr (kDa)	pI	Comments
IF-2	0136	96.1	-	Initiation factor-2
RbfA	0137	14.3	-	Ribosome binding factor
NusA	0460	40.7	-	Transcription elongation
GyrA	1027c	97.0	-	DNA gyrase
Pnp	1253	81.3	-	mRNA degradation
Tig	0193c	56.0	-	Trigger factor: aids protein secretion

Table 7.5: Cold-shock proteins detected within the genome of *Campylobacter jejuni* NCTC 11168

Analysis of the genome, in conjunction with 2D-PAGE studies (Section 5.13), revealed an absence of both CspA homologues, and a cold-shock response with induction of specific stress proteins. However, *C. jejuni* does possess some of the proteins that are expressed in *E. coli* as adaptation to cold-shock. Most of the proteins aid in transcription and translation. However, in *C. jejuni* no proteins are upregulated in response to cold-shock. These proteins (Table 7.5) may represent those proteins synthesised at 25°C (Section 5.13). However, at 4°C, protein synthesis is stopped after 30 min, yet the cells are still plateable, hence the mechanism of cold-shock survival in *C. jejuni* does not depend on *de novo* protein synthesis. It appears that *C. jejuni* survives at low temperature courtesy of a reduction in metabolic activity, staving off the accumulation of various cellular injuries, particularly membrane damage (Section 5.0).

7.6 pH stress response

Acid stress will be experienced by *C. jejuni* upon passage through the stomach. Additionally, phagocytes mediate their bactericidal activity courtesy of the combination of low pH and free radical bursts within phagolysosomes. Bile and pancreatic secretions make the small intestine (ileum, jejunum and duodenum) slightly alkaline. This contrasts with the acidity of the stomach. Furthermore, *C. jejuni* would encounter alkaline washes used in the poultry processing industry, such as TSP.

There are three transmembrane monovalent cation circuits involved in the maintenance of pH homeostasis:

(a) ***Proton circuit***: respiratory proton pump (export), entry routes (ATP synthetase, transport systems and flagellar motor).

(b) ***K^+ ion circuit***: uptake (KdpABCD) and efflux systems (KefABC).

(c) ***Na^+ ion circuit***: uptake, Na^+ channels and antiporters (NhaA, NhaB, ChaA)

Additionally, some general stress proteins, and specific systems for either acidic or alkaline stress are induced, such as deaminases and decarboxylases (Section 1.2.4). Analysis of the *C. jejuni* genome has revealed the presence of some proteins involved in pH homeostasis and acidic or alkaline stress (Table 7.6). The F_1F_0 ATPase and electron transport components are present in *C. jejuni* allowing generation and maintenance of a pmf. Acidic or alkaline pH would cause denaturation of proteins, hence the molecular chaperones such as GroES and GroEL would be induced. Proteases such as ClpP would degrade improperly folded proteins.

(i) Acid stress

It appears that *C. jejuni* does not possess an adaptive response to acid-shock (Cameron *et al.*, unpublished data), hence the absence of the GadABC system providing resistance to acid-shock in *E. coli* is no surprise. Mild acid tolerance in *C. jejuni* is likely to be passive due to the action of deaminases and glutamine accumulation (GlnHPQ). *C. jejuni* possesses deaminase activity (Leach *et al.*, 1997), and the basic end-products would aid neutralisation of intracellular acidity. Therefore, passage through the acidic environment of the stomach and colonisation of the intestine is likely to be facilitated by protection from the food matrix and the low infectious dose of this organism.

Protein	Cj number	Mr (kDa)	pI	Comments
GlnH	0817	28.9	-	Glutamine import
GlnP	0901	27.8	-	Glutamine import
GlnQ	0902	27.0	-	Glutamine import
GroES	1220	9.5	5.2	Heat-shock protein
GroEL	1221	57.9	4.9	Heat-shock protein
NhaA1	1654c	42.4	-	Na^+/H^+ antiporter
NhaA2	1655c	42.4	-	Na^+/H^+ antiporter
NapA †	1684c	44.0	-	Na^+/H^+ antiporter
ClpP	0192c	21.7	-	ATP-dependent Clp proteolytic subunit
F_0F_1 ATPase	0102-0108	-	-	ATP synthetase

Table 7.6: Acid or alkaline-shock proteins detected within the genome of *Campylobacter jejuni* NCTC 11168. † = proteins found by both me and the Sanger Centre, but only given a name based on homology by me

(ii) Alkaline stress

C. jejuni possesses a Na^+/H^+ antiporter, NhaA, that is involved in resistance to alkaline stress by importing H^+ ions and exporting Na^+ ions. No homologues to NhaB or ChaA were present, neither was there a homologue to NhaR, the regulator of NhaA expression in *E. coli*. *C. jejuni* possesses a number of decarboxylases, such as arginine

decarboxylase (SpeA; 0764), carboxynorspermidine decarboxylase (NspC; 1515c) and a putative decarboxylase (0768). The products of decarboxylases will be acidic and therefore would neutralise intracellular alkalinity. Whether these decarboxylases are constitutive or induced is unknown.

It is known that *C. jejuni* possesses an adaptive response to alkaline shock (Wu *et al.,* 1994) with the induction of GroES and GroEL. Furthermore, there is an adaptive response to TSP (Section 6.0) with the induction of at least 9 proteins. TSP acts primarily due to the high alkalinity of solutions. Some of the proteins in Table 7.6 may represent those proteins induced after TSP treatment.

7.7 Osmotic stress defence

Bacteria need to respond to both high and low osmotic stress, depending on the situation, such as entry into aquatic or marine systems, and survival on salted foods.

(i) High osmotic stress: As the external osmolarity increases, the osmolarity of the cytoplasm must increase to maintain turgor. Enzymes lose activity at high osmolarity, therefore adaptation occurs by accumulation of solutes compatible with enzyme function, such as, betaine, proline, ectoine and trehalose (Booth, 1999).

(ii) Low osmotic stress: An increase in external osmolarity results in outflow of water via aquaporins, resulting in turgor loss. This activates the constitutive TrkAEH K^+ uptake system, until turgor is restored. If K^+ is not available then a high K^+ affinity system (KdpABCD) is induced. In conjunction, glutamate is either imported or synthesised. Finally, compatible solutes are accumulated, whilst K^+ ions (KefA/B/C)

and glutamate are exported, reducing the salt concentration of the cytoplasm. Betaine is imported by either ProP and ProU (ProVWX) or synthesised by BetAB. Choline is imported by BetT. Trehalose is synthesised by OtsAB. Betaine and trehalose are degraded by BetX and TreA respectively (Booth, 1999).

C. jejuni possesses some of the machinery required to respond to such stress (Table 7.7).

Protein	Cj number	Mr (kDa)	pI	Comment
ProP †	0250c	49.2	-	Proline/ betaine/ ectoine importer
ProP †	0981c	46.9	-	Proline/ betaine/ ectoine importer
ProP †	1588c	48.5	-	Proline/ betaine/ ectoine importer
ProB	0097	29.2	-	Glutamate-5-kinase
ProWX †	0732	37.4	-	Proline/ betaine/ ectoine importer
ProV †	1538c	38.8	-	Proline/ betaine/ ectoine importer
PutP	1502c	54.2	-	Na^+/ proline symporter
KdpA	0676	31.5	-	K^+ importer
KdpB	0677	73.7	-	K^+ importer
KdpC	0678	15.3	-	K^+ importer
KdpD	0679	69.1	-	K^+ importer regulatory protein
KefB	1231	60.1	-	Glutathione regulated-K^+ exporter
KtrA	1284	23.6	-	K^+ uptake protein
KtrB	1283	49.5	-	K^+ uptake protein
DnaK	0759	67.3	4.8	Heat-shock protein
GroES	1220	9.5	5.2	Heat-shock protein
GroEL	1221	57.9	4.9	Heat-shock protein
ClpP	0192c	21.7	-	ATP-dependent Clp proteolytic subunit

Table 7.7: Proteins involved in protection against osmotic stress present within the genome *Campylobacter jejuni* NCTC 11168. † = proteins found by both me and the Sanger Centre, but only given a name based on homology by me

C. jejuni does not possess the necessary proteins to synthesise betaine or trehalose, however, the machinery for synthesis of proline (ProABCX) are present. Additionally, it appears that compatible solutes are accumulated by specific uptake systems, including three homologues of ProP and ProVWX which would play a role in importing proline. PutP is a proline permease, allowing proline to accumulate intracellularly. *C. jejuni* does not contain homologues of the stretch-activated

channels (MscL) and aquaporins (AqpZ) which respond to increase in turgor pressure, by allowing water (and ions) to flow out of the cell, restoring turgor pressure. However, there are plenty of uncharacterised putative OMPs present within the genome which may fulfil similar functions. In *H. pylori*, transcription of the *cbp*A-*hsp*R-*orf* and *gro*ESL operons is induced by osmotic stress (Spohn & Scariato, 1999). Similar regulation may occur in *C. jejuni*.

C. jejuni possesses two K^+ import systems (KdpABCD and KtrAB). The Kdp system is high affinity whilst the Ktr system may be analogous to the TrkAEH low affinity system. Such systems would be activated upon osmotic stress mediated by high salt concentrations such as marine ecosystems or salted foods.

7.8 Chemical and miscellaneous stresses

Many bacteria possess efflux systems which protect the cells from the deleterious effects of antibiotics, detergents and organic solvents. A number of such systems have been detected within the *C. jejuni* genome (Table 7.8).

AcrAB possibly causes the observed acridine orange efflux observed in Section 3.5. Other efflux systems (e.g. AcrDE, MsbA, NorA, Ebr and PmrA homologues) may contribute to antibiotic resistance observed in *C. jejuni* (Talsma *et al.*, 1999) and CFDA efflux (Section 3.4). Bcr promotes resistance to bicyclomycin and sulphathiazoles. *C. jejuni* possesses the ability to efflux various heavy metal ions such as Cu^{2+}, Zn^{2+}, Cd^{2+} and AsO_2^- via CopA, AtcU, CzcD, AcrDE, and ArsB (Silver & Phung, 1996). LytB promotes resistance to penicillins and plays a role in the stringent response (Mukherjee *et al.*, 1998), whilst a putative periplasmic β-lactamase is encoded by the genome. The genome encodes BacA, a protein involved in resistance to the antibiotic bacitracin.

Protein	Cj number	Mr (kDa)	pI	Comments
AcrA	0366c	114.0	-	Acriflavine/ drug efflux
AcrB	0367c	40.1	-	Acriflavine/ drug efflux
AcrR	0368c	24.2	-	Regulates AcrAB expression
Bcr †	0035c	44.1	-	Drug efflux: Bicyclomycin resistance
MsbA	0803	65.3	-	Drug efflux/ lipid export
AtcU †	1155c	88.5	-	Cu^{2+} exporter
CopA †	1161c	77.9	-	Cu^{2+} exporter
CzcD †	1163c	35.9	-	Cation efflux (Co^{2+}, Zn^{2+}, Cd^{2+})
ArsB	1187c	48.8	-	Arsenical membrane pump
AcrE	1032	27.8	-	Cation efflux (Co^{2+}, Zn^{2+}, Cd^{2+})
AcrD	1033	111.7	-	Cation efflux
??	0309c-0310c	11.5/ 14.2	-	Efflux pump
Ebr †	1173-1174	12.7	-	Efflux pump/ ethidium bromide resistance
PmrA †	1257c	42.8	-	Efflux pump
??	1375	47.3	-	Efflux pump
Bmr/NorA †	1375	47.3	-	Efflux pump/ quinolone resistance
NorA †	1687	47.5	-	Efflux pump/ quinolone resistance
BacA *	0205	29.8	-	Undecaprenol kinase (bacitracin resistance)
Ctc *	0311	19.5	-	General stress protein
MdaB	1545c	22.2	-	Modulator of drug activity
LytB	0894c	31.5	-	Lysis tolerance protein
?? *	0299	30.0	-	Periplasmic β-lactamase

Table 7.8: Efflux systems and other stress proteins found within the *Campylobacter jejuni* NCTC 11168 genome. ?? = unknown protein, † = proteins found by both me and the Sanger Centre, but only given a name based on homology by me, * = proteins found by the Sanger Centre only

7.9 Summary

It is evident from the increasing number of cases of *Campylobacter* enteritis in the U.K, that *C. jejuni* possesses the ability to survive the various stresses imposed during its journey through the food chain. This is despite a genome one-third the size of *E. coli*, and the ensuing coding limitations

Analysing the genome reveals that *C. jejuni* possesses the capability to respond to a number of stresses. A full complement of heat-shock proteins are present, probably regulated by a combination of HrcA and HspR. Surprisingly, given the ability of *C. jejuni* to survive at refrigeration temperature for prolonged periods, no

cold-shock response is present (Section 5.0). Due to the microaerophilic nature of the organism, it is perhaps unsurprising that a range of detoxification enzymes are present. It would appear that the PerR and Fur proteins regulate these enzymes. Although unable to synthesise compatible solutes with the exception of proline, *C. jejuni* possesses proteins that enable uptake of such compounds, perhaps explaining persistence in aquatic systems. *C. jejuni* does not have many proteins involved in pH stress responses. Possession of three possible Na^+/H^+ antiporters indicates the potential to survive moderate alkalinity, however there is little evidence of an inducible response to acidic pH. Some stationary phase/ starvation proteins are present, although the regulation of these proteins is presently unclear in the absence of an RpoS homologue. The necessary proteins required to launch a stringent response to both amino acid (SpoT, GppA) and carbon (CstA) starvation are present. A number of efflux systems are present, including AcrAB, presumably conferring resistance to antibiotics, heavy metal ions, detergents and hydrophobic solvents or disinfectants.

Therefore, *C. jejuni* possesses the protein machinery to withstand environmental stress, with the possible exception of cold-shock and acid-shock. The identification of these stress proteins will allow campylobacteriologists to unravel the secrets of *C. jejuni* survival in the environment and host, and perhaps enable development of better techniques for reducing the presence of this organism in the food chain.

Section 8

Final conclusions

8.0 Final conclusions

The response of *C. jejuni* to a number of stresses was investigated. Temperatures below 25°C (cold-shock), oxidative shock (aeration or peroxide stress) and chemical stresses (trisodium phosphate) challenge the ability of the bacterium to survive outside the intestinal tract and during poultry processing.

This study, and the literature (reviewed in Section 1.0) indicates that *C. jejuni* survives for considerable periods of time in the plateable state at reduced temperatures (particularly 4°C), even in the presence of other stresses. This is despite the absence of CspA homologues and protein synthesis being diminished within 30 min at 4°C. Nevertheless, under a microaerobic atmosphere the cells remain plateable for up to 13 days at 4°C, maintaining intact outer and cytoplasmic membranes, as well as possessing dehydrogenase and esterase activity. This poses some interesting questions. How can cells survive at 4°C in the absence of protein synthesis? How can coccoid formation occur in the absence of protein synthesis? How do the cells restart protein synthesis after temperature upshift? How do the cells revert from the non-plateable to the plateable state? The answers are not completely known, however, drawing on the data obtained in this study, and results published elsewhere, hypotheses can be put forward.

How can the cells survive at 4°C in the absence of protein synthesis?

The evidence indicates a gradual process whereby the cells experience a temporal spate of injuries (Figure 8.1), the severity of which depends upon the contact time with the stress. The rate of accumulation of the injuries is more rapid at higher incubation temperatures. Upon cold-shock, initially the ability to form colonies on selective agar is lost, followed by outer membrane damage, as the cells enter the non-

plateable state (unable to form colonies on non-selective plates). These cells can revert to the plateable state via temperature upshift and dilution into fresh broth (removal of 3-10 kDa inhibitor), confirming the presence of ABNC cells. Further stress results in damage to the cytoplasmic membrane, and loss of the ability to resuscitate. Prolonged entry into the non-plateable state results in gradual loss of dehydrogenase and esterase activity, and eventually coccoid formation. The gradual loss of physiological functions upon cold-shock in *C. jejuni*, is observed in other bacteria such as *S. typhimurium*, *Deleya aquamarina* and *Legionella pneumophila* (Yamamoto *et al.*, 1996, Joux *et al.*, 1997a,b, and Caro *et al.*, 1999), although in these studies, starvation stress was also applied.

Dodd *et al.,* (1997) forwarded a hypothesis that upon stress application, exponential phase cells, being more metabolically active than stationary phase cells, effectively 'commit suicide'. This occurs as a result of a free radical burst due to an imbalance between anabolism and catabolism. The time-dependent gradual loss of physiological function in *C. jejuni* is comparable to this hypothesis. Consider cells incubated at the non-growth permissive temperatures of 4 and 20°C. Due to temperature imposed enzyme reaction rate constraints, and lack of cell growth, metabolic imbalance will be greater in cells incubated at 20°C; hence the cells will produce a greater free radical burst. This is supported by the increased loss of plating ability, coccoid formation (Figure 5.1), and increased respiration rates at 20°C (Rollins & Colwell, 1986 and Hazeleger *et al.,* 1998) compared to lower temperatures. Such a theory could be examined by the measurement of free radicals generated at the different temperatures by free radical reaction with lucigenin visualised by luminometry (Aldsworth *et al.,* 1998).

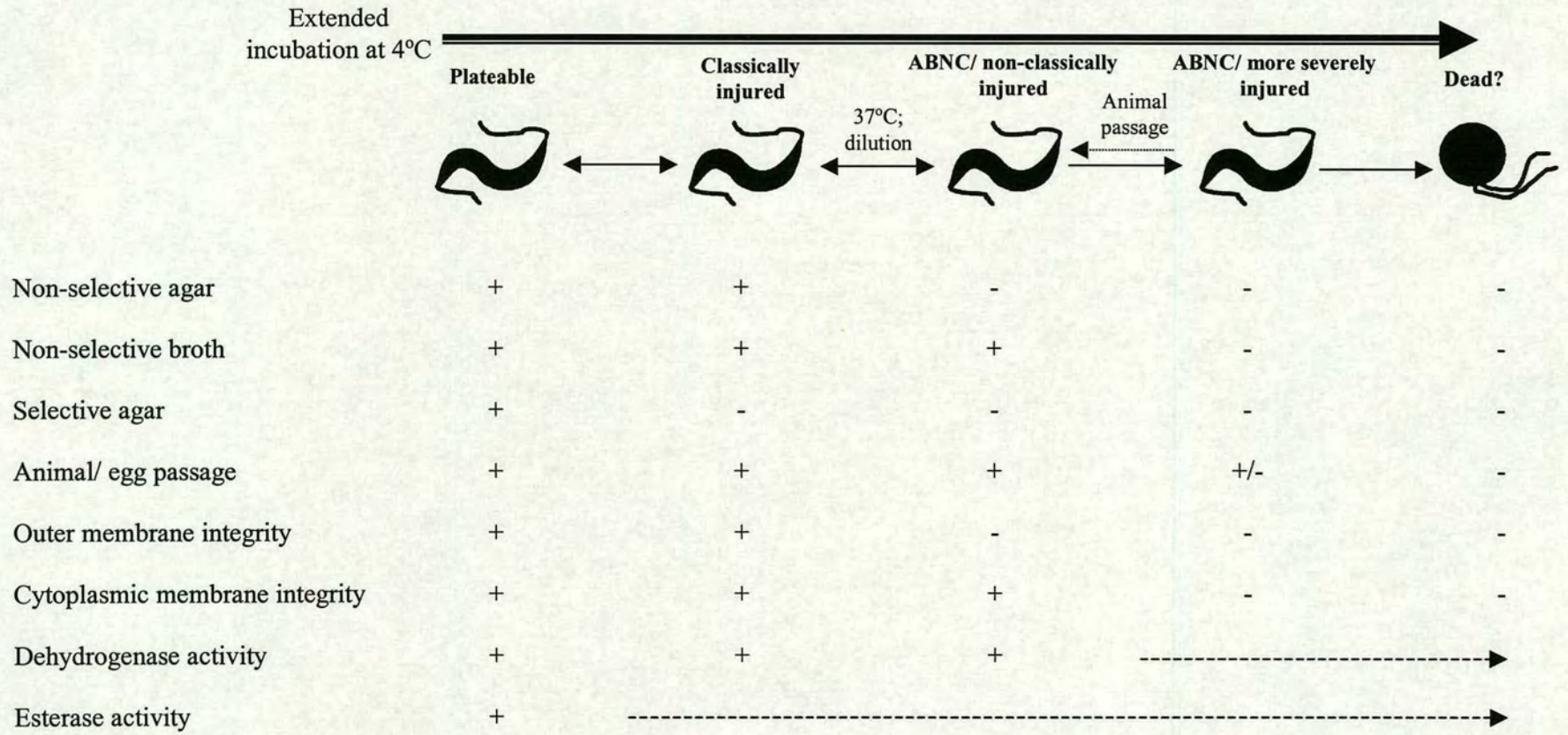


Figure 8.1: Diagrammatical representation of the known physiological changes experienced over prolonged periods of time by *Campylobacter jejuni* upon low temperature incubation. Dashed arrow indicates gradual loss of activity

How can coccoid formation occur in the absence of protein synthesis?

Coccoid formation occurs in the presence of chloramphenicol and irradiation at the same rate as controls in the absence of treatment (Figure 4.3; Hazeleger *et al.*, 1995 and Thomas *et al.*, 1999a). Therefore, coccoid formation is independent of DNA transcription and *de novo* protein synthesis, indicating a passive process.

Coccoid formation requires changes in cell wall structure, implying the involvement of cell wall modification enzymes (penicillin binding proteins or PBPs). This is supported by evidence indicating the temperature dependent nature of coccoid formation (Figure 5.1), and the observation that inactivating spiral cells by boiling or formaldehyde prevented coccoid formation (Table 4.2). Both conditions would inhibit enzyme function.

H. pylori is known to possess four PBPs (PBP-1A, PBP-2, PBP-3 and a 47 kDa PBP), present in different levels within spiral and coccoid cells (DeLoney & Schiller, 1999). Coccoid formation in *H. pylori* has been linked to the action of an endopeptidase (Costa *et al.*, 1999). It is known from the genome sequence of *C. jejuni*, that at least three PBPs are present (PBP-1A, PBP-2 and PBP-3). For coccoid formation to occur in the absence of protein synthesis, those enzymes responsible must already be present within the cell, prior to cold-shock. The stress could cause a reduction in overall metabolic activity. However, one of the degradative PBPs may retain activity, whilst the others are inactivated (PG-synthesising PBPs) thus reducing their levels. Hence, the net activity of the PBPs would determine coccoid formation, the rate of which (as for any enzyme reaction) would occur more rapidly at higher temperatures.

It is known that the thermophilic campylobacters (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) form coccoid cells much more rapidly than *C. fetus* subsp *fetus*, *C.*

fetus subsp *venerealis*, *C. sputorum* and *Arcobacter butzleri* (Karmali *et al.*, 1981 and Tenover & Fennell, 1991). Interestingly, according to Tenover & Fennel (1991) these members of the *Campylobacteriaceae* are either more aerotolerant (*A. butzleri*, *C. sputorum*) or can grow at lower growth temperatures e.g. 20-25°C (*C. fetus* subsp *fetus*, *C. fetus* subsp *venerealis* and *A. butzleri*). Could differences in the stability and/or activity of the enzymes (presumably PBPs) responsible for coccoid transformation be the cause of this phenomenon?

The actions of the three known PBPs could be examined by creating knockout mutants in *C. jejuni*. However, this may prove difficult; due to the necessity of PBPs in cell wall growth, the mutations may be lethal. Alternatively, inhibitors could be used to prevent the activity of specific PBPs, such as mecillinam inhibition of PBP-3. This would allow determination of the effect of loss of PBP-3 activity on coccoid formation. The proposed hypothesis would appear to classify the coccoid cell as a degenerate or moribund state. This is supported by the progressive injury theory of survival at low temperature. However, this may depend on the particular stress being applied, with coccoid cells formed under some conditions (4-10°C) being viable, whilst those formed under another set of conditions (20-30°C) being dead.

How can the cells restart protein synthesis after temperature upshift?

In *E. coli*, it is hypothesised that cold shock causes translation initiation to be transiently blocked, resulting in a decrease in polysomes, and a concomitant increase in 70S, 50S and 30S ribosomes, incapable of translation, hence mRNA gets stuck in the ribosomal machinery (Yamanaka, 1999). Cold shock proteins related to aiding translation are synthesised (IF-1, IF-2, RbfA, NusA, GyrA). Polynucleotide phosphorylase (Pnp) degrades mRNA, preventing blockage of the ribosomes, whilst

CsdA unwinds secondary structures which form in RNA at low temperature, stabilising the structure. Ribosome binding factor (RbfA) helps to initiate translation at low temperature (Yamanaka, 1999 and Phadtare *et al.,* 1999).

In *C. jejuni* there is an absence of protein synthesis at low temperature, hence although the genes for some of these proteins are present in the genome (Section 7.5) they are not synthesised. Perhaps this prevents translational blockage of ribosomes in the absence of CspA and CsdA plus the lack of synthesis of IF-1, IF-2, RbfA, NusA and GyrA. Resumption of protein synthesis after temperature upshift, may be due to the presence of ribosomes and proteins already present within the cell, perhaps becoming active again after the temperature upshift. Perhaps due to the rapid shutdown, ribosomes are not blocked and hence can begin synthesis immediately upon temperature upshift. This could be examined by metabolic labelling with [^{35}S]-methionine during the resuscitation period.

How do the cells revert from the non-plateable to the plateable state?

Reversal from the non-plateable to the plateable state occurs within a 4-7h lag period (Figure 5.5) dependent upon dilution into fresh broth and a temperature upshift to at least 37°C. A minimum of a three-fold dilution is required to completely remove the effects of a 3-10 kDa resuscitation inhibitor (Figure 5.9). Due to the size of the inhibitor, it would appear to be a peptide rather than a *N*-acyl homoserine lactone. Both are known to act as signalling molecules in various bacterial genera for functions such as, quorum sensing, competence, conjugation, and even virulence (Swift *et al.,* 1999). Loss of plating ability is linked to damage to the outer membrane, hence it would appear that the observed recovery after the 4-7h lag period is probably due to the repair of any membrane damage incurred during low temperature incubation. The

fact that resuscitation is prevented by addition of ampicillin and chloramphenicol indicates the process is at least partially dependent on peptidoglycan and protein synthesis. However, it is not known whether this occurs before or after membrane repair. The temporal kinetics of macromolecular synthesis during resuscitation can now be elucidated in a similar manner to starvation recovery in *S. aureus* (Clements & Foster, 1998). Furthermore, non-plateable cells formed in ABCD broth have a longer lag period than those formed in Brucella broth. Different nutrients or minerals present in Brucella broth may aid the resuscitation process. This could be investigated using ABCD broth as the basis and modulating the concentrations of various nutrients and minerals.

Significance of the data to the food industry

It is obvious that cold storage affords protection to *C. jejuni*, and other pathogens (Byrd *et al.,* 1991 and Smith *et al.,* 1994), even in the presence of other deleterious stresses. However, the dichotomy in survival at ambient temperatures (20-25°C) compared to refrigeration temperature (4°C), could be utilised within the poultry industry, with disinfectant washes performed at the higher temperatures. This is supported by the results concerning various chemical stresses at differing temperatures (Figures 6.3 and 6.4). Additionally, it is evident that aerobic packaging conditions would facilitate deterioration of *C. jejuni*. However, caution should be heeded; what reduces *C. jejuni* levels may increase levels of *Salmonella* or food spoilage organisms. A balance must be achieved.

Cells can adapt to sublethal concentrations of hydrogen peroxide and trisodium phosphate, enabling survival against lethal concentrations. Such conditions may be prevalent during poultry processing, for example, trisodium phosphate is used

as a chemical wash (Slavik *et al.,* 1994), hypochlorous acid produces free radicals inducing an oxidative stress response (Dukan & Touati, 1996), furthermore oxidative shock would be ubiquitous to *C. jejuni* outside its natural environment. However, due to the shutdown of protein synthesis at low temperature, it is questionable whether adaptation occurs. Studies require to be undertaken in this area. Upon refrigeration, the bacterial core temperature will gradually decrease from 37 to 4°C. The length of time this will take is unknown, however, perhaps the relevant stress proteins are synthesised during this period. It is known that protein synthesis (although reduced) occurs for at least 4h at 25°C (Figure 5.21.2), and Hazeleger *et al.,* (1998) demonstrated that at 4°C, *C. jejuni* possesses sufficient catalase activity to withstand reactive oxygen intermediates produced by its own respiratory metabolism.

The presence of recoverable non-plateable cells has obvious implications for the food, water and medical industries, in terms of the detection of this potentially infective state. Normal plating methods would be inadequate. It is worth noting that plating on selective media, as is usual in these industries, would underestimate the numbers of plateable bacteria present on the food surface (Figure 5.12). Similarly, selective enrichment in broths would preclude the detection of both non-plateable and injured, plateable *C. jejuni* cells. However, non-selective enrichment for at least 4-7h would allow repair of membrane damage to non-plateable cells, prior to selective enrichment or plating onto selective media, producing a better estimate of *C. jejuni* numbers. Given the low dilution (three-fold) required to eliminate the effects of the resuscitation inhibitor, it is unlikely that it plays a significant role in survival within aquatic or marine ecosystems, where dilutions greater than three-fold would occur immediately. Nevertheless, upon a refrigerated poultry surface significant

concentrations could accumulate, preventing resuscitation on the poultry surface (and detection). Ingestion of the food could provide the required stimuli for resuscitation, namely, dilution of the inhibitor and temperature upshift to at least 37°C. Although this is speculation, the increased incidences of *Campylobacter* food poisoning should stimulate further study.

Genome analysis has revealed some of the capabilities of *C. jejuni* to survive stress. Adaptive stress responses to heat shock (Konkel *et al.,* 1998), iron starvation (van Vliet *et al.,* 1998), oxidative shock (Section 6.0), and alkaline shock (Section 6.0; Wu *et al.,* 1994) have been demonstrated. Additionally, genome analysis reveals probable responses to osmotic stress and starvation/ stationary phase. Surprisingly, *C. jejuni* does not possess adaptive stress responses to cold-shock (Section 5.0), indicating the importance of low temperature incubation (4-10°C) in preventing injuries accumulated more rapidly at higher temperatures (20-30°C). There is no adaptive response to acid shock (Wu *et al.,* 1994 and Cameron *et al.,* unpublished), demonstrating the importance of protection by the food matrix in passage through the stomach.

This study has investigated the survival of *C. jejuni* to a variety of stresses that would be encountered within the poultry processing industry. The physiological response of *C. jejuni* to cold-shock has been elucidated in both the plateable and non-plateable states. Furthermore, this study provides conclusive evidence of *in vitro* resuscitation of metabolically active, non-plateable cells. Adaptive stress responses to oxidative shock (hydrogen peroxide) and trisodium phosphate (but not to cold-shock) were observed, with the synthesis of specific stress proteins. It is hoped that further

research into areas highlighted during this study will permit more efficient containment or elimination of this pathogen from poultry products.

Section 9

Appendix

9.0 Appendix: Analysis of the *Campylobacter jejuni* genome

The genome of *C. jejuni* NCTC 11168 has been sequenced by the Sanger Centre. Table 9.1 represents a systematic analysis of the proteins encoded by the genome using the Internet based search engine provided by the Sanger Centre (http://www.sanger.ac.uk/Projects/C_jejuni). It represents those proteins deemed by the software to provide the best-fit to the decoded *C. jejuni* sequence. All proteins designated within this table were personally searched by the author during the period of study. In no way should this table be assumed to be complete or correct. Although certain proteins were double-checked, the reader should view the list as a general guide to the biochemical and physiological capabilities of this organism. Permission was obtained from Julian Parkhill from the Sanger Centre to print this list. For the complete version created by the Sanger Centre, readers are referred to the above website.

Coding sequence	Protein
Cj0001	Chromosomal replication initiation protein (DnaA)
2 OX	**DNA polymerase 3 beta subunit (DnaN)**
3	DNA gyrase subunit B (GyrB)
4c	Putative periplasmic protein
5c	Putative molybdopterin containing oxidoreductase
6	Putative integral membrane protein
7	Glutamate synthase large chain (GltB)
8	?
9	Glutamate synthase small chain (GltD)
10c	Ribonuclease HII (RnhB)
11c	Putative non-specific DNA binding protein
12c	Non-haem binding protein; Rubrerythrin (RubY)
13	Dihydroxyacid dehydratase (IlvD)
14c	Putative integral membrane protein
15	?
16c	Putative transcriptional regulatory protein
17c	Putative ATP/GTP binding protein
18c	Small hydrophobic protein
19c	Methylaccepting chemotaxis protein (MCP)
20c OX	**Cytochrome C-551 peroxidase**
21c	Hypothetical protein
22c	Putative ribosomal pseudouridine synthase
23	Adenylosuccinate lyase (PurB)
24	Ribonucleoside diphosphate reductase alpha chain (NrdA)
25c	Proton-glutamate symport protein (GltP)
26c	?
27	CTP synthase (PyrG)
28	Single-strand DNA specific exonuclease (RecJ)

29	Cytoplasmic L-asparginase (AnsA)
30	?
31-32	Type IIS restriction/modification system
33	Putative integral membrane protein
34c	Putative periplasmic protein
35c DRUG	**Putative efflux protein (bicyclomycin resistance protein; Bcr)**
36	?
37c	Putative cytochrome c
38c	Putative membrane protein
39c	GTP-binding protein (TypA)
40-41	?
42	Flagellar hook assembly protein (FlgD)
43	Flagellar hook protein (FlgE)
44c	?
45c	Putative iron-binding protein
46	Transmembrane transport protein (pseudogene)
47c-52	?
53c	tRNA-5-methylaminomethyl-2-thiouridylate-methyltransferase (TrmU)
54c-56	?
57-58	Putative periplasmic proteins
59c	Flagellar motor switch protein (FliY)
60c	Flagellar motor switch protein (FliM)
0061c	**Sigma factor 28/ Flagellar sigma factor (RpoF/FliA)**
62c	Putative integral membrane protein
63c	MinD; cell division protein
64c	Flagellar biosynthesis protein (FlhF)
65c	7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase (FolK)
66c	3-dehydroquinate dehydratase (AroQ)
67	?
68	Protease IV (PspA)
69-71c	?
72c	Iron-binding protein (pseudogene)
73c	?
74c	Putative Fe-S protein
75c	Putative oxidoreductase Fe-S subunit
76c	L-lactate permease (LctP)
77c-79c	Cytolethal distending toxins A, B, C (CdtA, CdtB, and CdtC)
80	Putative membrane protein
81 STAT	**Cytochrome BD-II oxidase subunit I (CydA)**
82 STAT	**Cytochrome D ubiquinol oxidase subunit (CydB)**
83-84c	?
85c	Aspartate racemase
86c	Uracil-DNA glycosylase (Ung)
87	Aspartate-ammonia lyase (AspA)
88	Anaerobic C4-dicarboxylate transporter (DcuA)
89-91	Putative lipoproteins
92-93	Putative periplasmic proteins
94	50S ribosomal protein L21
95	50S ribosomal protein L27
96	Putative GTP-binding protein
97 OSM	**Glutamate-5-kinase (ProB)**
98	Methionyl-tRNA formyltransferase (Fmt)
99	Putative biotin (acetyl co-enzyme A carboxylase) synthetase (BirA)
100	ParA family ATPase
101	ParB family ATPase
102-108 ALK/ACID	**ATP synthase B, alpha, beta, gamma, delta, epsilon subunits (AtpACDGHFX)**
109-110	Putative biopolymer transporter (ExbBD)
111	Periplasmic protein
112	Colicin tolerance-like protein (TolB)
113	Peptidoglycan associated lipoprotein (Pal)
114	Putative periplasmic protein
115	Peptidyl-prolyl cis-trans isomerase (SlyD)
116	Malonyl coenzyme A-acyl carrier protein transacylase (FabD)
117	5'-methylthioadenosine/5'-adenosylhomocysteine nucleosidase (Pfs)
118-122	?
123c	Putative transcriptional regulator
124c	Putative membrane protein

125c HEAT	**DnaK suppressor protein (DksA)**
126c	?
127c	Acetyl coenzyme A carboxylase beta subunit (AccD)
128c	SuhB-like protein
129c	OMP surface antigen
130	Prephenate dehydrogenase (TyrA)
131	?
132	**UDP-3-O-[3-hydroxymyristoyl]-N-acetylglucosamine deacetylase (LpxC/EnvA)**
133	?
134	Homoserine kinase (ThrB)
135	?
136 COLD	**Translation initiation factor IF-2 (InfB)**
137 COLD	**Ribosome-binding factor A (RbfA)**
138	?
139	Endonuclease
140	?
141c-143c	ABC transporter system
144	Methyl-accepting protein (MCP)
145	?
146c OX	**Thioredoxin reductase (TrxB)**
147c OX	**Thioredoxin (TrxA)**
148c	?
149c	Homoserine dehydrogenase (Hom)
150c	Aspartate aminotransferase (AspB)
151c-152c	?
153c	rRNA/tRNA methyltransferase
154c	Putative methylase
155c	50S ribosomal protein L31
156c-160c	?
161c	Molybdenum co-factor biosynthesis protein (MoaA)
162-163c	?
164c	4-hydroxybenzoate octaprenyltransferase (Cytochrome C assembly factor; UbiA)
165	?
166	tRNA delta(2)-isopentenylpyrophosphate transferase (MiaA)
167c-168c	?
169c OX	**Fe-Superoxide dismutase (SodB)**
170-172c	?
173c-175c	Iron uptake ABC rtansport system
176c-177c	?
178c	Outer membrane haemin receptor
179-180	Biopolymer transporter (ExbBD)
181	TonB1 protein
182	Putative integral membrane protein
183	Transmembrane membrane protein
184c	Serine/threonine protein phosphatase
185c	PhnA proten
186c	?
187c	Phosphoribosylglycinamide formyl transferase (PurN)
188-189c	?
190c	Hypothetical protein
191c	Polypeptide deformylase (Def)
192c ACID	**ATP-dependent ClpP protease**
193c COLD	**Trigger factor (Tig)**
194	GTP cyclohydrolase I (FolE)
195	Flagellum specific ATP synthase (FliD)
196c	Amidophosphoribosyl transferase (DapB)
197c	Dihydropicolinate reductase (DapB)
198c	Helicase-like protein
199c-202c	?
203c	Putative transmembrane transport protein
204c	?
205c DRUG	**Undecaprenol kinase (BacA)/ Bacitracin resistance**
206	Threonyl-tRNA synthetase (ThrS)
207	Translation initiation factor IF-3 (InfC)
208	DNA modification methylase
209-222	?
223	Pseudogene (IgA protease family)

224	N-acetyl-gamma-glutamylphosphate reductase (ArgC)
225	Putative acetyltransferase
226	N-acetylglutamate kinase (ArgB)
227	Acetylornithine aminotransferase (ArgD)
228c	Protein-L-isoaspartate O-methyltransferase (Pcm)
229c	Putative acetyltransferase
230c	?
231c	Ribonucleoside diphosphate reductase B (NrdB)
232c	?
233c	Orolate phosphoribosyltransferase (PyrE)
Cj0234c	Ribosme recycling factor (Rrf)
235	Protein-export membrane protein (SecG)
236	?
Cj0237	Carbonic anhydrase (CynT)
238	?
239c	NifU-like protein
240c	Putative aminotransferase (NifS-homologue)
241c	Putative iron-binding protein
242c-243c	?
244	50S ribosomal protein L35
245	50S ribosomal protein L20
246c	Methyl accepting chemotaxis protein (MCP)
247c-249c	?
250c OSM	**Proline/betaine transporter (ProP)**
251c	Highly acidic protein
252c	Molybdenum co-factor biosynthesis protein (MoaC)
253c-254c	?
255c	Exodeoxyribonuclease (ExoA)
256	?
257	Diacylglycerol kinase (DgkA)
258	Putative helix-turn-helix motif protein
259	Dihydroorotase (PyrC)
260c-261c	?
262c	Methyl accepting chemotaxis protein (MCP)
263	?
264c	Biotin sulphoxide reductase 2 (BisC/ BmsA)
265c	Putative cytochrome c type, haem binding protein (TorC)
266c-268c	?
269c	Branched-chain aminoacid aminotransferase (IlvE)
270	Putative isomerase
271	Bacterioferritin comigratory protein (Bcp)
272	?
273	(3R)-hydroxymyristoyl-(acyl carrier protein) dehydratase (FabZ)
274	UDP-N-acetylglucosamine acyltransferase (LpxA)
275	**ATP-dependent Clp protease ATP-binding subunit (ClpX)**
276	Rod-shape determining protein (MreB)
277	Rod-shape determining protein (MreC)
278	?
279	Carbamoylphospahate synthase (large subunit) (CarB)
280	?
281c	Transaldolase (Tal)
282c	Phosphoserine phosphatase (SerB)
283c	CheW - purine binding chemotaxis protein
284c	CheA
285c	CheV
286c	?
287c	Transcription elongation factor (GreA)
288c	Lipid A-disaccharide synthase (LpxB)
289c	Major antigenic peptide (PEB3)
290c-292c	Phosphoglycerate transporter proteins (GlpT)
293 STAT	**Stationary phase survival protein (SurE)**
294	MoeB/ThiF family protein
295	Putative acetyltransferase
296c	Aspartate-1-decarboxylase precursor (PanD)
297c	Pantoate-beta-alanine ligase (PanC)
298c	2-methyl-2-oxobutanoate hydroxymethyl transferase (PanB)
299 DRUG	**Periplasmic beta-lactamase**

300c-303c	Molybdenum ABC transporter subunits (ModABC)
304c	Biotin synthesis protein (BioC)
305c	?
306c	8-amino-7-oxononoate synthase (BioF)
307	Adenosylmethionine-8-amino-7-oxononoate synthase (BioA)
308c	Dethiobiotin synthetase (BioD)
309c-310c DRUG	**Efflux protein**
311	**Ctc protein homologue**
312	Peptidyl tRNA hydrolase (Pth)
313	?
314	Diaminopimelate decarboxylase (LysA)
315	?
316	Chorismate mutase (PheA)
317	Histidinolphosphate aminotransferase (HisC)
318	Flagellar M-ring protein (FliF)
319	Flagellar motor switch protein (FliG)
320	Flagellar export protein (FliH)
321	1-deoxyxylulose-5-phosphate synthase (Dxs)
322 OX	**PerR (Peroxide stress regulator)**
323	?
324	Ubiquinone/menaquinone biosynthesis methyl transferase (UbiE)
325	Exodeoxyribonuclease VII (large subunit; XseA)
326	Phosphoserine aminotransferase (SerC)
327	?
328	3-oxoacyl-(acyl carrier protein) synthase (FabH)
329c	Fatty acid/phospholipid synthesis protein (PlsX)
330c	50S ribosomal protein L32
331c	?
332c	Nucleoside diphosphate kinase (Ndk)
333c	Ferredoxin (FdxA)
334 OX	**Alkylhydroperoxidase small subunit (AhpC)**
335	Flagellar biosynthesis protein (FlhB)
336	Flagellar motor rotation protein (MotB)
337	Motility protein (MotA)
338 OX	**DNA polymerase I (PolA)**
339c	Shikimate transport protein
340	Putative nucleotide hydrolase
341c	?
342c	Exinuclease ABC subunit A (UvrA)
343c-344c	?
345-347	Anthranilate synthase component I (TrpDEF)
348-349	Tryptophan synthase alpha, beta chains (TrpAB)
350	?
351	Flagellar switch protein (FliN)
352	?
353c STAT	**Guanosine pentaphosphate phosphohydrolase (GppA)**
354c	Ferredoxin (FdxB)
355c	Two component regulator
356c-357c	?
358 OX	**Cytochrome c peroxidase**
359c	?
360	Putative phospho-sugar mutase
361	Lipoprotein signal peptidase (LpsA)
362	?
363	Putative oxidoreductase
364	?
365c	OMP channel protein
366c-367c STAT	**Acriflavin resistance protein AB/D**
368c	**Transcriptional regulator (AcrR)**
369c	Ferredoxin-like integral membrane protein
370	30S ribosomal protein S21
371	Motility protein
372	?
373	Phosphoglycerate dehydrogenase (SerA)
374-376	?
377	Cell division control protein 48, AAA
378c-380c	?

381c	Orotidine 5' phosphate decarboxylase (PyrF)
382c	Transcription factor (NusB)
383c	6,7-dimethyl-8-ribitylumazine synthase(RibH)
384c	2-dehydro-3-deoxyphosphooctonate aldolase (KdsA)
385c	?
386	Putative GTP-binding protein
387	Shikimate kinase (AroK)
388	Tryptophanyl tRNA synthetase (TrpS)
389	Seryl tRNA synthetase (SerS)
390	TPR domain
391c	?
392c	Pyruvate kinase (Pyk)
393c	Putative oxidoreductase
394-397	?
398	Putative Glu-tRNA Gln amidotransferase (GatC)
399	?
400	**Fur protein (ferric uptake regulator)**
401	Lysyl tRNA synthetase (LysS)
402	Serine hydroxymethyl transferase (GlyA)
403-404	?
405	Shikimate 5-dehydrogenase (AroE)
406c	?
407	Prolipoprotein diacylglyceryl transferase (Lgt)
408-410	Fumarate reductase (FrdABC)
411-412	Putative ATP/GTP-binding proteins
413	?
414-415	Oxidoreductase subunits
416-421	?
422c	Putative helix-turn-helix motif protein
423	Putative integral membrane protein
424	Putative acidic periplasmic protein
425	?
426	ABC transporter, ATP-binding protein
427-428c	?
429c	Proline dipeptidase (PepQ)
430	?
431	Putative periplasmic ATP/GTP binding protein
432c	UDP-N-acetylmuramoylalanine-D-glutamate ligase (MurD)
433c	Phospho-N-acetylmuramoyl-pentapeptide transferase (MraY)
434	2,3-biphosphoglycerate independent phospho (Pgm)
435	3-oxoacyl-[acyl carrier protein] reductase (FabG)
436	?
437-439	Succinate dehydrogenase (SdhABC)
440c	Putative transcriptional regulator
441	Acyl carrier protein (AcpP)
442	3-oxoacyl-[acyl carrier protein] synthase (FabF)
443	Acetyl coenzyme A carboxylase carboxyltransferase subunit alpha (AccA)
444	Ton B dependent outer membrane receptor (pseudogene)
445	?
446	Ferric receptor (CfrA)
447	?
448c	Methyl accepting chemotaxis protein
449c	?
450	Ribosomal L28 protein
451	Ribulose phosphate 3-epimerase (Rep)
452 OX	**DNA polymerase III epsilon subunit (DnaQ)**
453	Thiamine biosynthesis protein (ThiC)
454c-459	?
460 COLD	**N utilisation substance protein A (NusA)**
461c-462	?
463	Zinc-protease-like protein
464	ATP-dependent DNA helicase (RecG)
465c	?
466	**Cyclic AMP receptor-like protein**
467-469	Amino acid ABC transporter
470	Elongation factor Tu (EF-Tu) (TufA)
471	50S ribosomal protein L33

472	Preprotein translocase (SecE) subunit
473	Transcription antitermination protein (NusG)
474	50S ribosomal protein L11
475	50S ribosomal protein L1
476	50S ribosomal protein L10
477	50S ribosomal protein L7/L12
478	**DNA-directed RNA polymerase beta subunit (RpoB)**
479	**RNA polymerase alpha subunit (RpoC)**
480c	Transcriptional regulator (KdgR)
481	Putative lyase
482-483	Altronate hydrolase (UxaA)
484	Transmembrane transporter protein
485	Putative oxidoreductase
486	Glucose/galactose transporter
487-488	?
489-490	Aldehyde dehydrogenase (Ald)
491	30S ribosomal protein S12
492	30S ribosomal protein S7
493	Elongation factor G (FusA)
494-497	?
498	Indole-3-glycerol phosphate synthase (TrpC)
499	Hit family protein
500	?
501	Ammonium transporter (Pseudogene)
502	?
503c	Ferrochelatase (HemH)
504c	?
505c	Putative aminotransferase (DegT family)
506c	Alanyl tRNA synthetase (AlaS)
507	MAF homologue (Maf)
508	Penicillin binding protein 1A (PBP-1A)
509c HEAT	**ClpB protein (HSP)**
510c	?
511	Carboxy-terminal protease
512	Phosphoribosylaminoimidazole-succinoc (PurC)
513	?
514	Phosphoribosylformylglycinamidine synthase (PurQ)
515	?
516	1-acyl-SN-glycerol-3-phosphate acyltransferase (PlsC)
517	CrcB protein homolog
518 HEAT	**HtpG (HSP)**
519-524	?
525c	Cell division protein (FtsI; PBP-3)
526	Flagellar basal protein (FliE)
527c	Flagellar basal body rod protein (FlgC)
528c	Flagellar basal body rod protein (FlgB)
529c	Aminodeoxychorismate lyase (PabC)
530	?
531	Isocitrate dehydrogenase (Icd)
532	Malate dehydrogenase (Mdh)
533-534	Succinyl-coA synthetase alpha, beta chains (SucCD)
535-538	2-oxoglutarate: accepto-oxidoreductase (OorABCD)
539-540	?
541	Octaprenyl-diphosphate synthase (IspB)
542	Glutamyl tRNA reductase (HemA)
543	Prolyl tRNA synthetase (ProS)
544	?
545	Porphobilinogen deaminase (HemC)
546	?
547	Flagellar protein (FlaG)
548	Flagellar hook associated protein 2 (FliD)
549	Flagellar protein (FliS)
550	?
551	Elongation factor P (EF-P)
552-557	?
558c OSM	**Gamma-glutamyl phosphate reductase (ProA)**
559 OX	**Thioredoxin reductase (TrxB)**/ oxidoreductase

560-561	?
562	Replicative DNA helicase (DnaB)
563-571	?
572	GTP cyclohydrolase II (RibA)
573	?
574-575	Acetohydroxy acid synthase large and small subunits (IlvHI)
576	UDP-3-O-(3-hydroxymyristoyl) N-acetyl deacetylase glucosamine (LpxD)
577c	S-adenosylmethionine tRNA ribosyltransferase-isomerase (QueA)
578c	Sec-independent protein translocase (MttB)
579c	?
580c	Oxygen-independent coporphyrinogen (HemN)
581 (VIR)	**Invasion associated protein/ NTPase (InvA)**
582	Aspartokinase (LysC)
583	?
584	DNA polymerase III delta/gamma subunit (HolB)
585	Dihydropteroate synthase (FolP)
586	DNA ligase (LigA)
587	?
588	Haemolysin (HlyA)
589	Riboflavin kinase (RibF)
590-594c	?
595c	Endonuclease III (Nth)
596	Cell binding factor 2 precursor (Cbf2; PEB-4)
597	Fructose biphosphate aldolase (Fba)
598-600	?
601c	Na^+/Cl^-dependent transporter
602c	?
603c	Thiol:disulphide interchange protein (DsbD)
604	?
605	N-acyl-L-amino acid amidohydrolase
606	MtrC protein
607	ABC transporter, ATP binding protein
608	Putative outer membrane protein
609-610c	?
611c	Alginate O-acetylation protein (AlgI)
612c	Ferritin (Cft)
613-616	Phosphate ABC transporter (PtsABCS)
617-618	Cj0617 family proteins
619-621	?
622	Transcription regulatory protein (HypF)
623-627	Hydrogenase expression/formation protein (HypABCDE)
628-630	?
631c (VIR)	**Ribonuclease II (VacB homolog of *H. pylori*)**
632	Ketol-acid reductoisomerase (IlvC)
633-635	?
636	Sun protein (Fmu protein)
637c	Protein-methionine sulphoxide reductase (MrsA)
638c	Inorganic pyrophosphatase (Ppa)
639c	Adenylate kinase (Adk)
640c	Aspartyl tRNA synthetase (AspS)
641	?
642	DNA repair protein (RecN)
643	Two component regulator protein
644	?
645	Putative secreted transglycosylase
646	Rare lipoprotein A (RlpA)
647-651	?
652	Penicillin binding protein-2 (PBP-2; PbpC)
653c	Aminopeptidase P
654c	Transporter transmembrane transport protein (pseudogene)
655c-660c	?
661c	GTP-binding protein (Era)
662c HEAT	**Heat-shock protein (HslU)**
663c HEAT	**Heat-shock protein (HslV)**
664c	50S ribosomal protein L9
665c	Argininosuccinate synthase (ArgG)
666c-668	?

669	ABC transporter, ATP-binding protein
670	**RNA polymerase sigma 54 (RpoN)**
671	Anaerobic C4-dicarboxylate transporter (DcuB)
672-675	?
676-679 OSM	**K^+-transporting ATPase (KdpABCD)**
680c OX	**Exinuclease ABC subunit B (UvrB)**
681-683	?
684	Primosomal protein N' (Replication factor; PriA)
685c	Putative sugar transferase
686	GcpE homologue
687	Flagellar basal body L-ring protein (FlgH)
688	Phosphate acetyltransferase (Pta)
689	Acetate kinase (AckA)
690c	Putative restriction/ modification enzyme
691-692	?
693	Fts-associated gene (FagA)
694-695	Cell division protein (FtsA)
696	Cell division protein (FtsZ)
697-698	Flagellar basal body protein (FlgG)
699c	Glutamine synthetase (GlnA)
700	?
701	Peptidase U32
702	Phosphoribosylaminoimidazole carboxylase (PyrE)
703	?
704	Glycyl tRNA synthetase alpha chain (GlyQ)
705-706	?
707	3-deoxy-D-manno-octulosonic acid transferase (KDO transferase) (KdtA)
708	Putative ribosomal pseudouridine synthase
709	Signal recognition protein (Ffh)
710	30S ribosomal protein L19
711	?
712	Putative 16SrRNA processing protein (RimM)
713	tRNA (guanine N1)-methyl transferase (TrmD)
714	50S ribosomal protein L19
715	Periplasmic transthyretin-like protein
716	Phospho-2-dehydro-3-deoxyheptanate aldolase
717	?
718 OX	**DNA polymerase III alpha chain (DnaE)**
719c	?
720c	Flagellin (FlaC)
721c	?
722c	Protoporphyrinogen oxidase (HemG)
723c HEAT	**Zinc-metallo protease (HtpX homologue)**
724	?
725c	Molybdopterin biosynthesis protein (Mog)
726c	Magnesium/cobalt transport protein (CorA)
727c	Periplasmic solute binding protein
728-729	?
730-732	ABC binding protein/permease transport system
733	?
734c	Histdine binding protein precursor (HisJ)
735-751	?
752	IS element transposase
753c	Iron transport protein (TonB3)
754	?
755	Ferric receptor (CfrA)
756	?
757 HEAT	**Heat-inducible transcriptional repressor (HrcA)**
758 HEAT	**GrpE**
759 HEAT	**DnaK (HSP70)**
760-761	?
762	Aspartate aminotransferase (AspB)
763c	Serine acetyltransferase (CysE)
764c ALK	**Arginine decarboxylase (SpeA)**
765c	Histidyl tRNA synthetase (HisS)
766c	Thymidylate kinase (Tmk)
767c	Lipopolysaccharide core biosynthesis protein (KdtB)

768c ALK	**Putative decarboxylase**
769c-772c	?
773c-774c	ABC transporter system
775c	Valyl tRNA synthetase (ValS)
776c	?
777 OX	**ATP-dependent helicase (UvrD)**
778	Major antigenic peptide (PEB2)
779 OX	**Thiol peroxidase (Tpx)**
780	Periplasmic nitrate reductase precursor (NapA)
781-782	Ferredoxin-type protein (NapGH)
783	Cytochrome C-type protein (NapB)
784	?
785	NapD protein
786-788	?
789	Polynucleotide adenyl transferase (PapS)
790	Formyltetrahydrofolate deformylase (PurU)
791c	Putative aminotransferase
792	?
793	Signal transduction histidine protein kinase
794	?
795c	UDP-MurNac-muramoylalanyl-D-glutamyl-2,6-diaminopimelate ligase (MurF)
796c	2-hydroxy-6-oxohepta-2,4-dienoate hydrolase
797c	?
798c	D-alanine-D-alanine ligase (DdlA)
799c	Holliday junction DNA helicase (RuvA)
800c	?
801 (VIR)	**Virulence factor (MviN)**
802	Cysteinyl tRNA synthetase (CysS)
803	Lipid export ABC transport protein (MsbA)
804	Dihydroorotate dehydrogenase (PyrD)
805	Zinc protease (PqqE)
806	Dihydrodipicolinate synthetase (DapA)
807	Short chain dehydrogenase
808c	?
809c	Putative hydrolase
810	NH_3-dependent NAD^+ synthetase (NadE)
811	Tetraacyldisaccharide-4-kinase (LpxK)
812	Threonine synthase (ThrC)
813	3-deoxy-manno-oculosonate cytidylyltransferase (KdsB)
814-816	?
817 ACID	**Glutamine binding periplasmic protein (GlnH)**
818-819	?
820c	Flagellar biosynthetsis protein (FliP)
821	UDP-N-acetylglucosamine pyrophosphorylase (GlmU)
822	Pantothenoate metabolism flavoprotein (Dfp)
823	?
824	Undecaprenyl diphosphate synthase (UppS)
825	Putative processing peptide
826	?
827	tRNA pseudouridine synthase A (TruA)
828	Threonine dehydratase (IlvA)
829c-830	?
831c	tRNA (uracil-5-)-methyltransferase (TrmA)
832c	?
833c	Oxidoreductase
834c	Ankyrin repeat containing periplasmic protein
835c	Aconitate hydratase (AcnB)
836	Methylated DNA-protein-cysteine methyltransferase (Ogt)
837c	?
838c	Methionyl tRNA synthetase (MetS)
839c	?
840c	Fructose-1,6-biphosphatase (Fbp)
841c	Putative ATP/GTP binding protein
842	?
843c	Lytic murein transglycosylase (Slt)
844c	?
845c	Glutamyl tRNA synthetase (GltX)

846	?
847	Phosphatidylserine decarboxylase (Psd)
848c-849c	?
850c	Transmembrane transport protein
851c-852c	
853c	Glutamate-1-semialdehyde 2,1-aminomutase (HemL)
854c	?
855	Methylenetetrahydrofolate dehydrogenase (FolD)
856	Signal peptidase I (LepP)
857c	Molybdopterin biosynthesis protein (MoeA)
858c	UDP-N-acetylglucosamine 1-carboxyvinyl transferase (MurA)
859c-860	?
861c-862c	Para-aminobenzoate synthetase (PabAB)
863c	Integrase-recombinase protein (XerD)
864	?
865	Putative disulphide oxidoreductase (DsbB)
866	Arylsulphatase (Ast; pseudogene)
867-871	?
872	Putative thiol:disulphide interchange protein (DsbA)
873	?
874c	Cytochrome c
875-881c	
882c	Flagellar biosynthesis protein (FlhA)
883c	?
884	30S ribosomal protein S15
885c	?
886c	Cell division protein (FtsK)
887c	Putative flagellin (FlaD)
888c	ABC transporter, ATP-binding protein
889c	Putative sensory transduction histidine kinase
890c	Phosphate regulon transcriptional regulator (PhoB)
891c	D-3-phosphoglycerate dehydrogenase (SerA)
892c	?
893c	30S ribosomal protein S1
894c	Lysis/penicillin tolerance protein (LytB)
895c	3-phosphoshikimate-1-carboxyvinyltransferase (AroA)
896c-897c	Phenylalanine tRNA synthetase alpha and beta subunits (PheST)
898	Hit family protein
899c	4-methyl-5(beta-hydroxyethyl)-thiazolemonophosphate (ThiJ)
900c	Small hydrophobic protein
901-902 ACID	**Glutamine ABC transporter (GlnPQ)**
903c	Na^+/H^+-dependent alanine transporter
904c	rRNA methylase (SpoU family)
905c	Alanine racemase (Alr)
906c-911c	?
912c	Cysteine synthase (CysM)
913c	DNA binding protein Hu homolog (HupB)
914c	*C. jejuni* invasion antigen (CiaB)
915c	Putative hydrolase
916c	?
917c STAT	**Carbon starvation protein A (CstA)**
918c	Ribosephosphate pyrophosphate kinase (PrsA)
919c-920c	Amino acid transporter
921c	PEB-1A (major cell-binding factor precursor; ABC transporter periplasmic solute binding protein)
922c	PEB-C (glutamine ABC transporter, ATP binding protein)
923c	Chemotaxis protein methyltransferase (CheR)
924c	Protein-glutamate methylesterase (CheB)
925	Ribose-5-phosphate isomerase (RpiB)
926	?
927	Adenine phosphoribosyltransferase (Apt)
928	?
929	Aminopeptidase (PepA)
930	GTP-binding protein
931c	Argininosuccinate lyase (ArgH)
932c	Phosphoenolpyruvate carboxykinase (PckA)
933c	Pyruvate carboxylase B subunit (PycB)
934c-935c	Na^+/Cl^- dependent transporter

936 ACID/ALK	**ATP synthase C chain (AtpE)**
937	?
938c	2-acylglycerophosphoethanolamine acyltransferase (Aas)
939c	?
940c ACID	**Glutamine ABC transporter, permease (GlnP)**
941c	?
942	Preprotein translocase (SecA) subunit
943c-944c	?
945c	Putative helicase
946c	?
947c	Putative hydrolase
948c-950c	?
951c	Methyl accepting chemotaxis protein
952c	?
953c	Phosphoribosylaminoimidazolecarboxamid (Pur9)
954c HEAT	**Putative DnaJ-like protein**
955c	Phosphoribosylglycinamide synthase (PurL)
956c OX	**Thiophene and furan oxidation protein (ThdF)**
957c	?
958c	60 kD inner membrane protein
959c	?
960c	Ribonuclease P (RnpA)
961c	50S ribosomal protein L34
962	Acetyltransferase
963-974	?
975	Haem-haemopexin utilisation protein B (Hxb1)
976-978	?
979c	Secreted nuclease
980	Putative peptidase
981c OSM	**Proline/betaine transporter (ProP)**
982c	Outer membrane protein
983-984	?
985c	Hippurate hydrolase (HipO)
986c-990c	?
991c	Glycerol-3-phosphate dehydrogenase (GlpC)
992c	Oxygen-independent coproporphyrinogen III oxidase (HemN)
993c	?
994c	Ornithine carbamoyltransferase (ArgF)
995c	Delta-aminoleuvilinc acid dehydratase (HemB)
996	GTP cyclohydrolase II (RibA)
997	Glucose inhibited division protein B (GidB)
998c-999c	?
Cj1000	Regulatory helix-turn-helix protein (LysR family)
1001	**RNA polymerase sigma factor (RpoD)**
1002c-1004c	?
1005c HEAT	**Cell division protein (FtsH)**
1006c-1007c	?
1008c	3-dehydroquinate synthase (AroB)
1009c	?
1010	Quenine tRNA-ribosyl transferase (Tgt)
1011-1012c	?
1013c	Cytochrome C biogenesis protein (CcsA)
1014c-1019c	High-affinity branched-chain amino acid ABC transporter (LivFGHJKM)
1020c	Putative cytochrome c
1021c-1022c	?
1023c	Aspartate-semialdehyde dehydrogenase (Asd)
1024c	Putative response regulator
1025-1026c	?
1027c COLD	**DNA gyrase subunit A (GyrA)**
1028c	Putative purine/pyrimidine phosphoribosyltransferase
1029c	Putative lipoprotein (MapA)
1030c	GTP-binding protein (LepA)
1031-1033 STAT	**Cation efflux (AcrB,D,F) Acriflavine resistance protein**
1034c HEAT	**Putative DnaJ-like protein**
1035c	Putative transferase
1036c	?
1037c	Pyruvate carboxylase A subunit (PycA)

1038	Cell division protein (FtsW)
1039	UDP-NAGlu-NAMur (pentapeptide) pyrophosphoryl undecaprenol NAGlu transferase (MurG)
1040c	?
1041c	Putative periplasmic ATP/GTP binding protein
1042c	AraC family regulatory proteins helix-turn-helix protein
1043c	Putative transferase
1044-1045	ThiGH
1046	Molybdopterin biosynthesis protein (MoeB)
1047c	?
1048c	Succinyl-diaminopimelate desuccinylase (DapE)
1049c	?
1050c	Putative transferase
1051c	Type I restriction enzyme
1052c	Mismatch repair protein (MutS)
1053c	?
1054c	UDP-N-acetylmuramate-alanine ligase (MurC)
1055-1057c	?
1058c	Inosine-5'-monophosphate dehydrogenase (GuaB)
1059c	Glu-tRNA Gln amidotransferase subunit A (GatA)
1060c	?
1061c	Isoleucyl tRNA synthetase (IleS)
1062	?
1063	Putative acetyltransferase
1064	Putative nitroreductase
1065-1066	Putative NAD(P)H nitroreductase (RdxA)
1067	CDP-alcohol phosphatidyl transferase (PgsA)
1068	? PDZ domain
1069	?
1070	Ribosomal protein S6
1071	Single strand DNA binding protein (Ssb)
1072	Ribosomal protein S18
1073 HEAT	**Lon ATP-dependent protease**
1074	Lipoprotein
1075	?
1076	Pyrroline-5-carboxylate reductase (ProC)
1077-1080c	?
1081c	Thiamine phosphate pyrophosphorylase (ThiE)
1082c	Thiamine biosynthesis protein (ThiD)
1083c	Putative nuclease
1084c	?
1085c	Transcription-repair coupling factor (Mfd)
1086c	?
1087c	ToxR-activated gene (TagE)
1088c	Folylpolyglutamate synthase (FolC)
1089c-1090c	?
1091c	Leucyl tRNA synthetase (LeuS)
1092c-1093c	Protein-export membrane protein (SecDF)
1094c	?
1095	Apolipoprotein N-acyltransferase
1096	S-adenosylmethionine synthetase (MetK)
1097	Na^+/H^+-glutamate symport protein (GltT)
1098	Aspartate carbamoyl transferase (PyrB)
1099	Oligoendopeptidase F (PepF)
1100	?
1101 OX	**DNA helicase II (UvrD)**
1102	tRNA pseudouridine synthase B (TruB)
1103 STAT	**Carbon storage regulator (CsrA)**
1104	?
1105	Small protein B (SmpB)
1106	Putative periplasmic thioredoxin
1107	?
1108 HEAT	**ATP-dependent Clp protease ATP-binding subunit (ClpA)**
1109	Leu/Phe-tRNA protein translocase (Aat)
1110c	Methyl accepting chemotaxis protein
1111c-1113c	?
1114c	CDP-diacylglycerol-serine O-phosphatidyl synthetase (PssA)
1115c	?

1116c HEAT	**Cell division protein (FtsH)**
1117c	Ribosomal protein methyltransferase (PrmA)
1118c	CheY protein
1119c	WlaM (Accessory colonisation factor)
1120c	WlaL (Polysaccharide biosynthesis protein)
1121c	WlaK (Aminotransferase)
1123c	WlaI (Acetyltransferase)
1124c	WlaH (UDP-galactophosphate transferase)
1125c	WlaG (Galactosyl transferase)
1126c	WlaF (Polysaccharide biosynthesis protein)
1127c	WlaE (Glycosyltransferase)
1128c	WlaD (Glycosyl transferase)
1129c	WlaC (Glycosyl transferase)
1130c	WlaB (ABC transporter, ATP-binding protein)
1131c	UDP-glucose 4-epimerase (GalE)
1133	LPS heptosyltransferase (WaaC)
1134	Lipid A biosynthsis acyltransferase (HtrB)
1135	LPS glucosyltransferase
1136	Galactosyltransferase
1137	Hypothetical protein
1138-1139c	Galactosyltransferases
1140	Hypothetical protein
1141	N-acetylneuraminic acid synthetase (NeuB3)
1142	UDP-N-acetylglucosamine-6-phosphate-2-epimerase (NeuC)
1143	Acylneuraminate cytidylyltransferase (NeuA)
1144c-1145c	?
1146c	Glucosyltransferase (WaaV)
1147c	?
1148	ADP-heptose-LPS heptosyltransferase I (WaaF)
1149c	Phosphoheptose isomerase (LpcA)
1150c	ADP-heptose synthase (WaaE)
1151c	ADP-L-glycero-D-mannoheptose-6-epimerase (WaaD)
1152c	Putative phosphatase
1153	Cytochrome C-553 precursor
1154c	Small hydrophobic protein
1155c	Cation-transporting ATPase
1156	Transcription termination factor rho (Rho)
1157 OX	**DNA polymerase III gamma subunit (DnaX)**
1158c-1160c	?
1161c DRUG	**Copper-transporting ATPase (CopA)**
1162c	?
1163c	Cation transporting protein
1164c-1166c	?
1167	L-lactate dehydrogenase (Ldh)
1168c	Alkaline phosphatase-like protein/ DadA
1169c	?
1170c	Outer membrane protein
1171c	Peptidyl-prolyl cis-trans isomerase (Ppi)
1172c-1174c DRUG	**Efflux protein**
1175c	Arginyl tRNA synthetase (ArgS)
1176c	?
1177c	Guanylate kinase (Gmk)
1178c	?
1179c	Flagellar biosynthesis protein (FliR)
1180c	ABC transporter, ATP binding protein
1181c	Elongation factor TS (EF-TS; Tsf)
1182c	30S ribosomal protein S2
1183c STAT	**Cyclopropane fatty acid synthase (Cfa)**
1184c-1186c	Ubiquinol cytochrome C oxidoreductase (PetABC)
1187c DRUG	**Arsenical pump membrane protein (ArsB)**
1188c	Glucose inhibited division protein A (GidA)
1189c	Signal transduction sensor protein
1190c	Methyl accepting chemotaxis protein
1191c	?
1192	C4-dicarboxylate transport protein (DctA)
1193	?
1194	Phosphate permease

1195c	Dihydroorotase (PyrC2)
1196c	NAD-dependent glycerol-3-phosphate dehydrogenase (GpsA)
1197c	Glu-tRNA Gln amidotransferase subunit B (GatB)
1198c	?
1199	Iron/ascorbate oxidoreductase
1200	OMP (??)
1201	5-methyltetrahydropteroyltriglutamate (MetE)
1202	5, 10 -methylenetetrahydrofolate reductase (MetF)
1203c	?
1204c ACID/ALK	**ATP synthase A chain (AtpB)**
1205c	ATP-dependent DNA repair protein (RadA)
1206c	Cell division protein, FtsY homologue
1207c	Putative lipoprotein thioredoxin
1208	?
1209	YmdA protein
1210	DedA family protein
1211	DNA transfer protein/ competence locus
1212c	Ribonuclease BN (Rbn)
1213c	Glycolate oxidase subunit D (GlcD)
1214c-1216c	?
1217c	YfiH (??)
1218c	Riboflavin synthase alpha chain (RibC)
1219c	?
1220 HEAT	**GroES**
1221 HEAT	**GroEL**
1222c	Two component histidine kinase sensor protein
1223c	Putative regulatory protein (CiaR)
1224	Putative iron-binding protein
1225	?
1226c	Putative histidine kinase sensor protein (CpxA)
1227c	Response regulator (OmpR)
1228c HEAT	**Serine protease (HtrA)**
1229 STAT	**Co-chaperone-curved DNA binding protein (CbpA)**
1230 HEAT	**Heat-shock transcriptional regulator (HspR)**
1231 OSM	**KefB/KefC: glutathione-regulated K^+/H^+ antiporter**
1232	?
1233	Phosphoglycolate phosphatase
1234	Glycyl tRNA synthetase beta chain (GlyS)
1232-1236c	?
1237c	Putative phosphatase
1238c	Pyridoxyl phosphate synthase (PdxJ)
1239	Pyridoxyl phosphatebiosynthesis protein (PdxA)
1240c	?
1241	Putative transmembrane transport protein
1242	?
1243	Uroporphyrinogen decarboxylase (HemE)
1244c-1245c	?
1246c OX	**Exinuclease ABC subunit C (UvrC)**
1247c	?
1248	GMP synthase (GuaA)
1249	?
1250	Phosphoribosylamine-glycine ligase (PurD)
1251-1252	?
1253 COLD	**Polyribonucleotide nucleotidyltransfer phosphorylase (Pnp)**
1254	?
1255	4-oxalocrotonate tautomerase
1256c	?
1257c DRUG	**Multidrug resistance pump**
1258	Putative phosphotyrosine protein phosphatase
1259	Major outer membrane protein (MOMP; PorA)
1260c HEAT	**DnaJ (HSP)**
1261 HEAT	**Response regulator protein (RacR)**
1262 HEAT	**Histidine protein kinase (RacS)**
1263	Recombination protein (RecR)
1264c-1267c	Ni/Fe-hydrogenase B-type cytochrome (HydABCD)
1268c	?
1269c	N-acetylmuramoyl-L-alanine amidase (AmiA)

1270c	?
1271c	Tyrosyl tRNA synthetase (TyrS)
1272c STRIN	**Pentaphosphate guanosine-3'-pyrophosphate synthetase (ppGpp; SpotT)**
1273c	**Putative DNA-directed RNA polymerase omega chain (RpoZ)**
1274c	Uridylate kinase (PyrH)
1275c	?
1276c	Cell division membrane protein (FtsX)
1277c	Cell division protein (FtsE)
1278c	?
1279c	Putative fibronectin domain containing lipoprotein
1280c	Putaive ribosomal pseudouridine synthase
1281	?
1282	Rod shape determining protein (RodA; MrdB)
1283-1284 OSM	**K^+ uptake protein (KtrAB)**
1285c	?
1286c	Uracil phosphoribosyltransferase (Upp)
1287c	Malate oxidoreductase
1288c	Glutamyl tRNA synthetase (GltX2)
1289	??
1290c	Biotin carboxylase (AccC)
1291c	Biotin carboxyl carrier protein (AccB)
1292c ACID	**Deoxycytidine triphosphate deaminase (Dcd)**
1293	Putative sugar nucleotide epimerase/ dehydratase
1294	Putative aminotransferase (DegT family)
1295-1298	?
1299	Acyl carrier protein (Acp2)
1300-1302	?
1303	Beta-ketoacyl-ACP synthase III (FabH2)
1304	Acyl carrier protein (Acp3)
1305-1306	Cj0617 family proteins
1307	D-alanine activating enzyme
1308	Acyl carrier protein (Acp4)
1309	?
1310	Cj0617 family protein
1311	Acetylneuraminate cytidyltransferase (NeuA2)
1312	Putative flagellar protein
1313	Flagellar protein
1314c	HisF protein (cyclase)
1315c	Amidotransferase (HisH)
1316c	Hypothetical protein
1317	Sialic acid synthase (NeuB)
1318	Cj1318 family protein
1319	Nucleotide sugar transferase
1320	Aminotransferase (DegT family)
1321	WlaI protein (acetyltransferase)
1322-1326	?
1327	Sialic acid synthase (NeuB2)
1328	N-acetylneuraminic acid synthetase (NeuC2)
1329	Putative sugar-phosphate nucleotide transferase
1330	?
1331-1332	Post-translational flagellin modification protein (PtmAB)
1333-1337	Cj1318 family proteins
1338c	Flagellin B (FlaB)
1339c	Flagellin A (FlaA)
1340c-1341c	?
1342c-1343c	Cj1318 family proteins
1344c	O-sialoglycoprotein endopeptidase (Gcp)
1345c	?
1346c	1-deoxy-D-xylulose-5-phosphate reductase (Dxr)
1347c	Phosphatidate cytidyl transferase (CdsA)
1348c-1349c	?
1350	Molybdopterin-guanine dinucleotide biosynthesis (MobA)
1351 VIR	**Phospholipase A (PldA)**
1352-1355	Enterochelin uptake system (CeuBCDE)
1356	?
1357c-1358c	Putative periplasmic cytochrome c
1359	Polyphosphate kinase (Ppk)

1360	Putative proteolysis tag for 10S RNA
1361c	?
1362	Holliday junction DNA helicase (RuvB)
1363c	Acid membrane antigen A (AmaA)
1364c	Fumarate hydratase (FumC)
1365c	Putative secreted serine protease
1366c	Glucosamine-fructose-6-phosphate aminotransferase (GlmS)
1367c	Putative nucleotidyltransferase
1368	?
1369	Xanthine/uracil permease
1370	Nucleotide phosphoribosyl transferase (Gpt)
1371	Periplasmic protein (VacJ homologue)
1372-1374	?
1375 DRUG	**Multidrug efflux transporter**
1376-1377c	?
1378c	L-seryl tRNA selenium transferase (SelA)
1379	Selenocysteine-specific elongation factor (SelB)
1380-1381	?
1382c	Flavodoxin (FldA)
1383c-1384c	?
1385 OX	**Catalase (KatA)**
1386	Ankyrin repeat containing protein
1387c	Helix trun helix motif protein
1388	?
1389	Transmembrane transport protein (pseudogene)
1390-1391	?
1392-1393	Cystathione beta-lyase (MetC)
1394	Fumarate lyase
1395-1397	?
1398	Ferrous iron transport protein B (FeoB)
1399c	Hydrogenase (HydA2)
1400c	Enoyl-acyl carrier protein reductase (FabI)
1401c	Triose phosphate isomerase (TpiA)
1402c	Phosphoglycerate kinase (Pgk)
1403c	Glyceraldehyde-3-phosphate dehydrogenase (GapA)
1404-1406	?
1407c	Phospho-sugar mutase
1408	Flagellar biosynthesis protein (FliL)
1409	Holo-ACP synthase (AcpS)
1410c	?
1411c	Cytochrome P450
1412c	?
1413c-1414c	Putative polysaccharide (capsular) modification protein
1415c	Adenylsulphate kinase (cysC)
1416c	Glucose-1-phosphate cytidylyltransferase
1417c-1418c	Hypothetical proteins
1419c-1420c	Putative methylases
1421c-1422c	Putative sugar transferase
1423c	Mannose/glucose-1-phosphate guanyl/thimidyl transferase
1424c	Phosphoheptose isomerase (GmhA2)
1425c	Putative sugar kinase
1426c	?
1427c	Putative sugar-nucleotide epimerase/dehydratase
1428c	Fucose synthetase (Fcl)
1429c	?
1430c	DTDP-4-dehydrorhamnose 3,5-epimerase (RfbC)
1431c	?
1432c-1434c	Sugar transferases
1435c	?
1436c	Putative aminotransferase
1437c	Histidinol phosphate aminotransferase
1438c	Sugar transferase
1439c	UDP-galactopyranose mutase (Glf)
1440c	Sugar transferase
1441c	UDP-glucose 6-dehydrogenase (KfiD)
1442c	?
1443c	KpsF protein

1444c	Polysialic acid transport protein (KpsD)
1445c	Capsule polysaccharide export inner membrane protein (KpsE)
1446c-1447c	Polysialic acid transporter, ATP-binding protein (KpsT)
1448c	Polysialic acid transport protein (KpsM)
1449c	?
1450	Putative ATP/GTP binding protein
1451	dUTPase (Dut)
1452-1454	?
1455	Peptide chain release factor-2 (RF-2) (PrfB)
1456c-1457c	?
1458c	Thiamine monophosphate kinase (ThiL)
1459c-1460	?
1461	Putative DNA methylase
1462	Flagellar P-ring protein (FlgI)
1463-1465	?
1466	Flagellar hook associated protein I (FlgK)
1467-1469	?
1470-1474	Putative type II secretion system FED proteins
1475c	?
1476c	Pyruvate-flavodoxin oxidoreductase (NifJ)
1477c	Phosphoglycolate phosphatase (Gph)
1478c	Outer membrane fibronectin binding protein (CadF)
1479c	30S ribosomal protein S9
1480c	50S ribosomal protein L13
1481c	ATP-dependent nuclease (AddA)
1482c-1486c	?
1487c-1490c	Cytochrome C oxidase (CBB3-type) (CcoNOPQ)
1491c	Two component regulator
1492c	Two component sensor
1493c	?
1494c	Carbamoylphosphate synthase small chain (CarA)
1495c-1497c	?
1498c	Adenylosuccinate synthetase (PurA)
1499-1501	?
1502c OSM	**Proline permease (PutP)**
1503c	Proline dehydrogenase (PutA)
1504c	Selenide, water dikinase (SelD)
1505c	?
1506c	Methyl accepting chemotaxis protein
1507c	?
1508c-1512c	Formate dehydrogenase (FdhABCD)
1513c-1514c	?
1515c ACID	**Carboxynorspermidine decarboxylase (NspC)**
1516c	Periplasmic Cu oxidoreductase
1517	Molybdopterin converting factor (MoaD)
1518	Molybdopterin converting factor (MoaE)
1519	Molybdopterin cofactor biosynthesis protein (MoeA2)
1520-1527	?
1528	Transmembrane transport protein (pseudogene)
1529c	Phosphoribosylformylglycinamide cyclo-ligase (PurM)
1530	?
1531	Diaminopimelate epimerase (DapF)
1532	Putative periplasmic protein (Cj153)
1553c	Putative helix turn helix motif protein
1534c VIR	**Neutrophil-activating protein (bacterioferritin; NapA);**
1535c	Glucose-6-phosphate isomerase (Pgi)
1536c	UTP-glucose-1-phosphate uridylyltransferase (GalU)
1537c	Acetyl coA synthetase (AcsA)
1538c OSM	**Osmoprotection protein (ProV)**
1539c	Sulphate transport system permease (CysT)
1540	?
1541	Lactam utilisation protein (LamB homolog)
1542-1544c	?
1545c DRUG	**Modulator of drug activity B (MdaB)**
1546	?
1547	Outer membrane lipoprotein (Blc protein homologue)
1548c	NADP-dependent alcohol dehydrogenase

1549c-1553c	Type I restriction enzyme system (RSM proteins)
1554c-1560	?
1561	**Transcriptional regulator (ArsR family)**
1562	?
1563c	Transcriptional regulator
1564	Methyl accepting chemotaxis protein
1565c	Paralysed flagellum protein (PflA)
1566c-1579c	NADH-ubiquinone oxidoreductase (NuoABCDEFGHIJKLMN)
1580c-1584c	Oligopeptide ABC transporter (DppABCDF)
1585c	Putative oxidoreductase
1586	Flavohaemoprotein (HmpA)
1587	ABC transporter ATP binding protein
1588c OSM	**Proline/betaine transporter (ProP)**
1589	?
1590 COLD	**Translation initiation factor, IF-1 (InfA)**
1591	50S ribosomal protein L36
1592	30S ribosomal protein S12
1593	30S ribosomal protein S11
1594	30S ribosomal protein S4
1595	**DNA-directed RNA polymerase alpha chain (RpoA)**
1596	50S ribosomal protein L17
1597	ATP phosphoribosyltransferase (HisG)
1598	Histidinol dehydrogenase (HisD)
1599	Imidazoleglycerol phosphate dehydratase (HisB)
1600	Amidotransferase (HisH)
1601	Phosphoribosylformine-5-amidoimidazo (HisA)
1602	?
1603	Cyclase (HisF)
1604	Phosphoribosyl-AMP cyclohydrolase (HisI)
1605c	Tetrahydropyridine-2-carboxylase (DapD)
1606c	ATP/GTP binding protein (Mrp homologue)
1607	?
1608	Response regulator
1609	Sulphate adenylyltranferase
1610	Phosphatidyl glycerophosphatase A (PgpA)
1611	30S ribosomal protein S20
1612	Peptide chain release factor-1 (RF-1) (PrfA)
1613	?
1614-1617	Haemin uptake system (ChuABCD)
1618	?
1619	Alpha-ketoglutarate permease (KgtP)
1620	A/G-specific adenine glycosylase (MutY)
1621	?
1622	Riboflavin-specific deaminase (RibD)
1623	?
1624c	L-serine dehydratase (SdaA)
1625c	Serine transporter (SdaC)
1626c-1627c	?
1628-1629	Biopolymer transport system (ExbBD)
1630	TonB2 transport protein
1631-1633c	?
1634c	Chorismate synthase (AroC)
1635c	Ribonuclease III (Rnc)
1636c	Ribonuclease H (RnhA)
1637c	?
1638	DNA primase (DnaG)
1639	NifU-like protein
1640	?
1641	UDP-N-acetylmuramoylalanyl-D-glutamate 2,6-diamiligase (MurE)
1642	AmaA (Acid membrane antigen A)
1643	?
1644	Geranyltransftransferase (IspA)
1645	Transketolase (Tkt)
1646-1648	ABC Transporter (IamAB)
1649-1650	?
1651c	Methionine aminopeptidase (Map)
1652c	Aspartate-Glutamate racemase (MurI)

1653c	?
1654c ALK	**Na+/H+ antiporter (NhaA2)**
1655c ALK	**Na+/H+ antiporter (NhaA1)**
1656c-1658	?
1659	Periplasmic protein p19
1660	?
1661-1663	ABC transporter
1664	Periplasmic thioredoxin
1665	Putative lipoprotein thioredoxin
1666	?
1667c	RepA homologue
1668c	?
1669c	DNA ligase (Lig)
1670c-1671c	?
1672c	Enolase (Eno)
1673c OX	**RecA**
1674	?
1675	Flagellar biosynthesis protein (FliQ)
1676	UDP-N-acetylenolpyruvoylglucosamine reductase (MurB)
1677-1680c	?
1681c	CysQ protein
1682c-1683c	Citrate synthase (GltA)
1684c ALK	**Na^+/H^+ antiporter (NapA)**
1685c	Biotin synthetase (BioB)
1686c STAT	**DNA topoisomerase I (TopA)**
1687	Putative efflux protein
1688c	Preprotein translocase subunit (SecY)
1689c-1708c	50S ribosomal protein L16, L18, L6, L5, L24, L14, L29, L16, L22, L2, L23, L4, L3; 30S ribosomal protein S5, S8, S14, S17, S3, S19, S10
1709c	Putative ribosomal pseudouridine synthase
1710c	?
1711c	Dimethyl adenosine transferase (KsgA)
1712	Purine nucleoside phosphorylase (PunB)
1713-1714	?
1715	Putative acetyltransferase
1716c-1717c	3-isopropylmalate dehydratase large and small subunits (LeuCD)
1718c	3-isopropylmalate dehydrogenase (LeuA)
1719c	2-isopropylmalate synthase (LeuB)
1720	?
1721c	Putative outer membrane protein
1722-1725	?
1726c	Homoserine o-succinyl transferase (MetA)
1727c	O-acetyl homoserine sulphydrylase (MetY)
1728c	?
1729c	Flagellar hook protein (FlgE2)
1730	?
1731c	Cross over junction endodeoxyribouclease (RuvC)

Table 9.1: Proteins identified in the genome sequence of *Campylobacter jejuni* NCTC 11168. ACID = acid stress, ALK = alkaline stress, COLD = cold-shock, DRUG = efflux systems, HEAT = heat-shock, OSM = osmotic stress, OX = oxidative shock, VIR = virulence and STAT = stationary phase

Section 10

Bibliography

10.0 Bibliography

Aho, M., Kurki, M., Rautelin, M. & Kosunen, T.U. (1989) Waterborne outbreak of *Campylobacter* enteritis after outdoors infantry drill in Utti, Finland. Epidemiology and Infection, 103 (1), 133-141

Aldsworth, T.G., Sharman, R.L., Dodd, C.E.R. & Stewart, G.S.A.B. (1998) A competitive microflora increases the resistance of *Salmonella typhimurium* to inimical processes: evidence for a suicide response. Applied and Environmental Microbiology, 64 (4), 1323-1327

Alexander, E., Pham, D. & Steck, T.R. (1999) The viable-but-nonculturable condition is induced by copper in *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. Applied and Environmental Microbiology, 65, 3754-3756

Allos, B.M. & Blaser, M.J. (1995) *Campylobacter jejuni* and the expanding spectrum of related infections. Clinical Infectious Diseases, 20, 1092-1101

Allos, B.M. (1997) Association between *Campylobacter* infection and Guillain-Barre syndrome. Journal of Infectious Disease, 176 (S2), S125-S128

Alm, R.A., Guerry, P. & Trust, T.J. (1993) The *Campylobacter* σ^{54} *fla*B flagellin promoter is subject to environmental regulation. Journal of Bacteriology, 175 (14), 4448-4455

Amano, K. & Shibata, Y. (1992) Structural studies of peptidoglycans in *Campylobacter* species. Microbiology and Immunology, 36 (9), 961-967

Andersen, A.P., Elliott, D.A., Lawson, M., Barland, P., Hatcher, V.B. & Puszkin, E.G. (1997) Growth and morphological transformations of *Helicobacter pylori* in broth media. Journal of Clinical Microbiology, 35 (11), 2918-2922

Andersson, Y., de Borg, B. & Studahl, G. (1997) Waterborne *Campylobacter* in Sweden: The cost of an outbreak. Water Science and Technology, 35 (11-12), 11-14

Arana, I., Muela, A., Iriberri, S., Egea, L. & Barcina, I. (1992) Role of hydrogen peroxide in loss of culturability mediated by visible light in *Escherichia coli* in a freshwater ecosystem. Applied and Environmental Microbiology, 58, 3903-3907

Arana, I., Pocino, M., Muela, A., Fernandez-Astorga, A. & Barcina, I. (1997) Detection and enumeration of viable but non-culturable transconjugants of *Escherichia coli* during the survival of recipient cells in river water. Journal of Applied Microbiology, 83 (3), 340-346

Arimi, S.M., Fricker, C.R. & Park, R.W.A. (1988) Occurrence of 'thermophilic' campylobacters in sewage and their removal by treatment processes. Epidemiology and Infection, 101, 279-286

Baillon, M-L.A., van Vliet, A.H.M., Ketley, J.M., Constantinidou, C. & Penn, C. (1999) An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. Journal of Bacteriology, 181 (16), 4798-4804

Baleux, B., Caro, A., Lesne, J., Got, P., Binard, S. & Delpeuch, B. (1998) Survie et maintien de la virulence de *Salmonella* Typhimurium VNC exposée simultanément à trois facteurs stressants expérimentaux. Oceanologica Acta, 21 (6), 939-950

Barcina, I., Lebaron, P. & Vives-Rego, J. (1997) Survival of allochthonous bacteria in aquatic systems: a biological approach. FEMS Microbiology Ecology, 23, 1-9

Barer, M.R., Gribbon, L.T., Harwood, C.R. & Nwoguh, C.E. (1993) The viable but non-culturable hypothesis and medical bacteriology. Reviews in Medical Microbiology, 4, 183-191

Barer, M.R. (1997) Viable but non-culturable and dormant bacteria: time to resolve an oxymoron and a misnomer. Journal of Medical Microbiology, 46, 629-631

Bej, A.K., Mahbubani, M.H. & Atlas, R.M. (1991) Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods. Applied and Environmental Microbiology, 57 (2), 597-600

Benaïssa, M., Babin, P., Quellard, N., Pezennec, L., Cenatiempo, Y. & Fauchere, J.L. (1996) Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form. Infection and Immunity, 64 (6), 2331-2335

Berglund, D.L., Taffs, R.E. & Robertson, N.P. (1987) A rapid analytical technique for flow cytometric analysis of cell viability using calcofluor white M2R. Cytometry, 8, 421-426

Berry, E.D. & Foegeding, P.M. (1998) Cold temperature adaptation and growth of microorganisms. Journal of Food Protection, 60 (12), 1583-1594

Betts, R.P., Bankes, P. & Banks, J.G. (1989) Rapid enumeration of viable micro-organisms by staining and direct microscopy. Letters in Applied Microbiology, 9, 199-202

Beumer, R.R., de Vries, J. & Rombouts, F.M. (1992) *Campylobacter jejuni* non-culturable coccoid cells. International Journal of Food Microbiology, 15, 153-163

Biosca, E.G., Amaro, C., MarcoNoales, E. & Oliver, J.D. (1996) Effect of low temperature on starvation-survival of the eel pathogen, *Vibrio vulnificus* biotype 2. Applied and Environmental Microbiology, 62 (2), 450-455

Birdsell, D.C. & Cota-Robles, E.H. (1967) Production and ultrastructure of lysozyme and ethylenediaminetetraacetate-lysozyme spheroplasts of *Escherichia coli*. Journal of Bacteriology, 93 (1), 427-437

Blankenhorn, D., Phillips, J. & Slonczewski, J.L. (1999) Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. Journal of Bacteriology, 181 (7), 2209-2216

Blankenship, L.C. & Craven, S.E. (1982) *Campylobacter jejuni* survival in chicken meat as a function of temperature. Applied and Environmental Microbiology, 44 (1), 88-92

Blaser, M.J., Hardesty, H.L., Powers, B. & Wang, W-L., L. (1980) Survival of *Campylobacter fetus* subsp. *jejuni* in biological milieus. Journal of Clinical Microbiology, 11 (4), 309-313

Blaser, M.J., Smith, P.F., Wang, W.L.L. & Hoff, J.C. (1986) Inactivation of *Campylobacter jejuni* by chlorine and monochloramine. Applied and Environmental Microbiology, 51, 307-311

Blaser, M.J. (1997) Epidemiologic and clinical features of *Campylobacter jejuni* infections. The Journal of Infectious Diseases, 176 (suppl 2), S103-S105

Blum, H., Beier, H. & Gross, H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis, 8 (2), 93-99

Bode, G., Mauch, F. & Malfertheiner, P. (1993) The coccoid form of *Helicobacter pylori*. Criteria for their viability. Epidemiology and Infection, 111, 483-490

Bolton, F.J., Coates, D. & Hutchinson, D.N. (1984) The ability of *Campylobacter* media supplements to neutralise photochemically induced toxicity and hydrogen peroxide. Journal of Applied Bacteriology, 56 (1), 151-157

Booth, I.R. & Louis, P. (1999) Managing hypoosmotic stress: aquaporins and mechanosensitive channels in *Escherichia coli*. Current Opinion in Microbiology, 2 (2), 166-169

Booth, I.R. (1999) Adaptation to extreme environments, Chapter 28, p652-671. *In.* Biology of the Prokaryotes, Lengeler, J.W, Drews, G, & Schlegel, H.G (eds), Blackwell Science

Boucher, S.N., Slater, E.R., Chamberlain, A.H.L. & Adams, M.R. (1994) Production and viability of coccoid forms of *Campylobacter jejuni*. Journal of Applied Bacteriology, 77, 303-307

Boucher, S.N., Chamberlain, A.H.L. & Adams, M.R. (1998) Enhanced survival of *Campylobacter jejuni* in association with wood. Journal of Food Protection, 61 (1), 26-30

Bovill, R.A. & Mackey, B.M. (1997) Resuscitation of 'non-culturable' cells from aged cultures of *Campylobacter jejuni*. Microbiology, 143, 1575-1581

Bracikowski, J.P., Hess, I.E. & Rein, M.F. (1984) *Campylobacter* osteomyelitis. Southern Medical Journal, 177 (2), 1611-1613

Brás, A.M., Chatterjee, S., Wren, B.W., Newell, D.G. & Ketley, J.M. (1999) A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonisation. Journal of Bacteriology, 181 (10), 3298-3302

Bremer, P.J., Osborne, C.M., Kemp, R.A. & Smith, J.J. (1998) Survival of *Listeria monocytogenes* in sea water and effect of exposure on thermal resistance. Journal of Applied Microbiology, 85, 545-553

Brock, F.M. & Murray, G.E. (1987) Minicell formation by *Campylobacter jejuni*. Canadian Journal of Microbiology, 33, 465-470

Buck, G.E., Parshall, K.A. & Davis, C.P. (1983) Electron microscopy of the coccoid form of *Campylobacter jejuni*. Journal of Clinical Microbiology, 18 (2), 420-421

Buswell, C.M., Herlihy, Y.M., Keevil, C.W., Marsh, P.D. & Leach, S.A. (1998) Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. Applied and Environmental Microbiology, 64 (2), 733-741

Buswell, C.M., Herlihy, Y.M., Keevil, C.W., Marsh, P.D. & Leach, S.A. (1999) Carbon load in aquatic ecosystems affects the diversity and biomass of water biofilm consortia and the persistence of the pathogen *Campylobacter jejuni* within them. Society for Applied Microbiology Symposium, 28, 161S-167S

Butler, R.C., Lund, V. & Carlson, D.A. (1987) Susceptibility of *Campylobacter jejuni* and *Yersinia enterocolitica* to UV radiation. Applied and Environmental Microbiology, 53 (2), 375-378

Buzoleva, L.S. (2000) Periodic culturing of nonculturable *Yersinia pseudotuberculosis* forms. Bulletin of Experimental Biology and Medicine, 4, 374-376

Byrd, J.J., Xu, H-S. & Colwell, R.R. (1991) Viable but nonculturable bacteria in drinking water. Applied and Environmental Microbiology, 57 (3), 875-878

Byrd, J.J. & Colwell, R.R. (1993) Long term survival and plasmid maintenance of *Escherichia coli* in marine microcosms. FEMS Microbiology Ecology, 12 (1), 9-14

Cappelier, J.M., Lazaro, B., Rossero, A., Fernandez-Astorga, A. & Federghi, M. (1997) Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable *Campylobacter jejuni* cells. Veterinary Research, 28, 547-555

Cappelier, J.M. & Federghi, M. (1998) Mises en évidence l'état viable non cultivable chez *Campylobacter jejuni*. Revue de Médicinie Véterinaire, 149 (4), 319-326

Cappelier, J.M., Magras, C., Jouve, J.L. & Federghi, M. (1999a) Recovery of viable but non-culturable *Campylobacter jejuni* cells in two animal models. Food Microbiology, 16, 375-383

Cappelier, J.M., Minet, J., Magras, C., Colwell, R.R. & Federghi, M. (1999b) Recovery in embryonated eggs of viable but nonculturable *Campylobacter jejuni* cells and maintenance of ability to adhere to HeLa cells after resuscitation. Applied and Environmental Microbiology, 65 (11), 5154-5157

Caro, A., Got, P., Lesne, J., Binard, S. & Baleux, B. (1999) Viability and virulence of experimentally stressed nonculturable *Salmonella typhimurium*. Applied and Environmental Microbiology, 65 (7), 3229-3232

Castro, H.P., Teixeira, P.M. & Kirby, R. (1997) Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze drying. Journal of Applied Microbiology, 82 (1), 87-94

Cellini, L., Allocati, N., Angekucci, D., Iezzi, T., DiCampli, E. & Marzio, L. (1994) Coccoid *Helicobacter pylori* not culturable in vitro reverts in mice. Microbiology and Immunology, 38 (11), 843-850

Cellini, L., Robuffo, I., Di Campli, E., Di Bartolomeo, S., Taraborelli, T. & Dainelli, B. (1998) Recovery of *Helicobacter pylori* ATCC43504 from a viable but not culturable state: regrowth or resuscitation? APMIS, 106, 571-579

Cha, M.K. & Kim, I.H. (1999) Thioredoxin in the periplasmic space of *Escherichia coli* as a physiological electron donor to periplasmic thiol peroxidase. Journal of Biochemistry and Molecular Biology, 32 (2), 168-172

Chan, F.T.H., Stringel, G. & MacKenzie, A.M.R. (1983) Isolation of *Campylobacter jejuni* from an appendix. Journal of Clinical Microbiology, 18 (2), 422-424

Chen, J. & Koopman, B. (1997) Effect of fluorochromes on bacterial surface properties and interaction with granular media. Applied and Environmental Microbiology, 63 (10), 3941-3945

Cho, J-C. & Kim, S-J. (1999) Viable, but non-culturable, state of a green fluorescence protein-tagged environmental isolate of *Salmonella typhi* in groundwater and pond water. FEMS Microbiology Letters, 170, 257-264

Chou, S.P., Dular, R. & Kasatiya, S. (1983) Effect of ferrous sulphate, sodium metabisulfite, and sodium pyruvate on survival of *Campylobacter jejuni*. Journal of Clinical Microbiology, 18 (4), 986-987

Chynoweth, R.W., Hudson, J.A. & Thomas, K. (1998) Aerobic growth and survival of *Campylobacter jejuni* in food and stream water. Letters in Applied Microbiology, 27, 341-344

Clavero, M.R.S., Monk, J.D., Beuchat, L.R., Doyle, M.P. & Bracket, R.E. (1994) Inactivation of *Escherichia coli* O157:H7, Salmonellae, and *Campylobacter jejuni* in raw ground beef by gamma irradiation. Applied and Environmental Microbiology, 60 (6), 2069-2075

Clements, M.O. & Foster, S.J. (1998) Starvation recovery of *Staphylococcus aureus* 8325-4. Microbiology, 144, 1755-1763

Colwell, R.R., Brayton, P.R., Grimes, D.J., Roszak, D.B., Huq, S.A. & Palmer, L.M. (1985) Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for the release of genetically engineered microorganisms. Bio/Technology, 3, 817-820

Colwell, R.R., Brayton, P., Herrington, D., Tall, B., Huq, A. & Levine, M.M. (1996) Viable but non-culturable *Vibrio cholerae* O-1 revert to a cultivable state in the human intestine. World Journal of Microbiology & Biotechnology, 12, 28-31

Costa, K., Bacher, G., Allmaier, G., Dominguez-Belo, M.G., Engstrand, L., Falk, P., de Pedro, M.A. & del Portillo, F.G. (1999) The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. Journal of Bacteriology, 181 (12) 3710-3715

Craig, J.E., Boyle, D., Francis, K.P. & Gallagher, M.P. (1998) Expression of the cold-shock gene *csp*B in *Salmonella typhimurium* occurs below a threshold temperature. Microbiology, 144, 697-704

Curtis, L.M., Patrick, M. & Blackburn, C.D. (1995) Survival of *Campylobacter jejuni* in foods and comparison with a predictive model. Letters in Applied Microbiology, 21 (3), 194-197

Darken, M.A. (1962) Absorption and transport of fluorescent brighteners by microorganisms. Applied Microbiology, 10, 387-393

Davey, H.M. & Kell, D.B. (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: The importance of single-cell analyses. Microbiological Reviews, 60 (4), 641-698

De Boer, P.A.G., Cook, W.R. & Rothfield, L.I. (1990) Bacterial cell division. Annual Review of Genetics, 24, 249-274

Deere, D., Porter, J., Edwards, C. & Pickup, R. (1995) Evaluation of the suitability of *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol, (diBA-$C_4(3)^-$), for the flow cytometric assessment of bacterial viability. FEMS Microbiology Letters, 130, 165-170

DeLoney, C.R. & Schiller, N.L. (1999) Competition of various β-lactam antibiotics for the major penicillin-binding proteins of *Helicobacter pylori*: antibacterial activity and effects on bacterial morphology. Antimicrobial Agents and Chemotherapy, 43 (11), 2702-2709

DeMélo, M.A., Gabbiani, G. & Péchère, J-P. (1989) Cellular events and intracellular survival of *Campylobacter jejuni* during infection of Hep-2 cells. Infection and Immunity, 57, 2214-2222

Demple, B. (1991) Regulation of bacterial oxidative stress genes. Annual Review of Genetics, 25, 315-337

Diaper, J.P. & Edwards, C. (1994) The use of fluorogenic esters to detect viable bacteria by flow cytometry. Journal of Applied Bacteriology, 77 (2), 221-228

Dodds, C.E.R., Sharman, R.L., Bloomfield, S.F., Booth, I.R. & Stewart, G.S.A.B. (1997) Inimical processes: bacterial self-destruction and sub-lethal injury. Trends in Food Science & Technology, 8, 238-241

Doig, P., Yao, R., Burr, D.H., Guery, P. & Trust, T.J. (1996) An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. Molecular Microbiology, 20 (4), 885-894

Dover, N., Higgins, C.F., Carmel, O., Rimon, A., Pinner, E. & Padan, E. (1996) Na^+-induced transcription of *nha*A, which encodes an Na^+/H^+ antiporter in *Escherichia coli*, is positively regulated by *nha*R and affected by *hns*. Journal of Bacteriology, 178 (22), 6508-6517

Doyle, M.P. & Roman, D.J. (1981) Growth and survival of *Campylobacter fetus* subsp. *jejuni* as a function of temperature and pH. Journal of Food Protection, 44 (8), 596-601

Doyle, M.P. & Roman, D.J. (1982a) Response of *Campylobacter jejuni* to sodium chloride. Applied and Environmental Microbiology, 43 (3), 561-565

Doyle, M.P. & Roman, D.J. (1982b) Sensitivity of *Campylobacter jejuni* to drying. Journal of Food Protection, 45 (6), 507-510

Dukan, S. & Touati, D. (1996) Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. Journal of Bacteriology, 178 (21), 6145-6150

Dukan, S., Lévi, Y. & Touati, D. (1997) Recovery of culturability of an HOCl-stressed population of *Escherichia coli* after incubation in phosphate buffer: resuscitation or regrowth? Applied and Environmental Microbiology, 63 (11), 4204-4209

Duncan, S., Glover, L.A., Killham, K. & Prosser, J.I. (1994) Luminescence-based detection of activity of starved and viable but nonculturable bacteria. Applied and Environmental Microbiology, 60 (4), 1308-1316

Dykes, G.A. & Withers, K.M. (1999) Sub-lethal damage of Listeria monocytogenes after long-term chilled storage at 4°C. Letters in Applied Microbiology, 28 (1), 45-48

Easton, J. (1996) Fate and transport of Campylobacters in soil arising from farm practices, p461-465. *In*, *Campylobacters*, *Helicobacters*, and Related Organisms. Newell, D.G, Ketley, J, & Feldman, R.A (eds), New York, Plenum

Eaton, K.A., Catrenich, C.E., Makin, K.M. & Krakowka, S. (1995) Virulence of coccoid and bacillary forms of *Helicobacter pylori* in gnotobiotic piglets. The Journal of Infectious Diseases, 171, 459-462

Effendi, I. & Austin, B. (1995) Dormant unculturable cells of the fish *pathogen Aeromonas salmonicida*. Microbial Ecology, 30 (2), 183-192

Ekweozor, C.C., Nwoguh, C.E. & Barer, M.R. (1998) Transient increases in colony counts observed in declining populations of *Campylobacter jejuni* held at low temperature. FEMS Microbiology Ecology, 158, 267-272

Enroth, H. & Engstrand, L. (1996) Egg passage of rod-shaped and coccoid forms of *Helicobacter pylori*: preliminary studies. Helicobacter, 1 (3), 183-186

Etchegaray, J-P. & Inouye, M. (1999) CspA, CspB, and CspG, major cold shock proteins of *Escherichia coli*, are induced at low temperature under conditions that completely block protein synthesis. Journal of Bacteriology, 181 (6), 1827-1830

Farkas, J. (1998) Irradiation as a method for decontaminating food - A review. International Journal of Food Microbiology, 44, 189-204

Farr, S.B. & Kogoma, T. (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiological Reviews, 55 (4), 561-585

Fearnley, C., Cawthraw, S.A., Ayling, R.D. & Newell, D.G. (1996) The failure of viable but non-culturable *Campylobacter jejuni* to colonise one-day-old chicks. *In*, *Campylobacters*, *Helicobacters*, and Related Organisms. Newell, D.G, Ketley, J, & Feldman, R.A (eds), New York, Plenum

Federighi, M., Tholozan, J.L., Cappelier, J.M., Tissier, J.P. & Jouve, J.L. (1998) Evidence of non-coccoid viable but non-culturable *Campylobacter jejuni* cells in microcosm water by direct viable count, CTC-DAPI double staining, and scanning electron microscopy. Food Microbiology, 15, 539-550

Ferguson, G.P. (1999) Protective mechanisms against toxic electrophiles in *Escherichia coli*. Trends in Microbiology, 7 (6), 242-247

Field, L.H., Headley, V.L., Payne, S.M. & Berry, L.J. (1986) Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. Infection and Immunity, 54 (1), 126-132

Flahaut, S., Hartke, A., Giard, J.C. & Auffray, Y. (1997) Alkaline stress response in *Enterococcus faecalis*: Adaptation, cross-protection, and changes in protein synthesis. Applied and Environmental Microbiology, 63 (2), 812-814

Flahaut, S., Laplace, J.M., Frére, J. & Auffray, Y. (1998) The oxidative stress response in *Enterococcus faecalis*: relationship between H_2O_2 tolerance and H_2O_2 stress proteins. Letters in Applied Microbiology, 26, 259-264

Foster, J.W. (1991) *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. Journal of Bacteriology, 173 (21), 6896-6902

Foster, J.W. (1999) When protons attack: microbial strategies of acid adaptation. Current Opinion in Microbiology, 2 (2), 170-174

Fouz, B., Toranzo, A.E., Marco-Noales, E. & Amaro, C. (1998) Survival of fish-virulent strains of *Photobacterium damselae* subsp. *damselae* in seawater under starvation conditions. FEMS Microbiology Letters, 168, 181-186

Fritz, L. & Triemer, R.E. (1985) A rapid simple technique utilizing calcofluor white M2R for the visualisation of dinoflagellate thecal plates. Journal of Phycology, 21, 662-664

Gaidenko, T.A. & Price, C.W. (1998) General stress transcription factor σ^B and sporulation transcription factor σ^H each contribute to survival of *Bacillus subtilis* under extreme growth conditions. Journal of Bacteriology, 180 (14), 3730-3733

Geilhausen, T.D. (1996) *Campylobacter* and *Salmonella* contamination of fresh chicken meat, p 105-108. *In*, *Campylobacters*, *Helicobacters*, and Related Organisms. Newell, D.G, Ketley, J, & Feldman, R.A (eds), New York, Plenum

Ghezzi, J.I. & Steck, T.R. (1999) Induction of the viable but nonculturable condition in *Xanthomonas campestris* pv. *campestris* in liquid microcosms and sterile soil. FEMS Microbiology Ecology, 30 (3), 203-208

Goldstein, J., Pollitt, N.S. & Inouye, M. (1990) Major cold shock protein of *Escherichia coli*. Proceedings of the National Academy Sciences USA, 87, 283-287

Gourmelon, M., Touati, D., Pommepuy, M. & Cormier, M. (1997) Survival of *Escherichia coli* exposed to visible light in seawater: analysis of *rpo*S-dependent effects. Canadian Journal of Microbiology, 43 (11), 1036-1043

Grant, K.A. & Park, S.F. (1995) Molecular characterisation of *kat*A from *Campylobacter jejuni* and generation of a catalase-deficient mutant of *Campylobacter coli* by interspecific allelic exchange. Microbiology, 141, 1369-1376

Graumann, P.L. & Marahiel, M.A. (1999) Cold shock proteins CspB and CspC are major stationary-phase-induced proteins in *Bacillus subtilis*. Archives in Microbiology, 171, 135-138

Griffiths, P.L. (1993) Morphological changes of *Campylobacter jejuni* growing in liquid culture. Letters in Applied Microbiology, 17, 152-155

Hall, H.K., Karem, K.L. & Foster, J.W. (1995) Molecular responses of microbes to environmental pH stress. Advances in Microbial Physiology, 37, 229-264

Hallock, F.A. (1959) The coccoid stage of vibrios. Transactions of the American Microscopical Society, 78, 231-237

Hammond, S.M., Lambert, P.A. & Rycroft, A.N. (1984) The bacterial cell surface. p 1-226. 1st edition, Croom Helm Ltd., London

Hanninen, M.L., Niskanen, M. & Korhonen, L. (1998) Water as a reservoir for *Campylobacter jejuni* infection in cows studied by serotyping and pulsed-field gel electrophoresis (PFGE). Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health, 45 (1), 37-42

Harris, N.V., Weiss, N.S. & Nolan, C.M. (1986) The role of poultry and meats in the etiology of *Campylobacter jejuni/coli* enteritis. American Journal of Public Health, 76, 407-411

Harvey, P., Fearnley, C., Newell, D., Hudson, M. & Leach, S. (1996) Coccal cell switching and the survival and virulence of *C. jejuni* at high oxygen tensions. *In*, *Campylobacters*, *Helicobacters*, and Related Organisms. Newell, D.G, Ketley, J, & Feldman, R.A (eds), New York, Plenum

Harvey, P. & Leach, S. (1998) Analysis of coccal cell formation by *Camplyobacter jejuni* using continuous culture techniques, and the importance of oxidative stress. Journal of Applied Microbiology, 85, 398-404

Haughland, R.P. (1999) Molecular Probes: Handbook of fluorescent probes and research chemicals. 7th edition

Hazeleger, W.C., Arkesteijn, C., Toorop-Bouma, A. & Beumer, R. (1994) Detection of the coccoid form of *Campylobacter jejuni* in chicken products with the use of the polymerase chain reaction. International Journal of Food Microbiology, 24, 273-281

Hazeleger, W.C., Janse, J.D., Koenraad, P.M.F.J., Beumer, R.R., Rombouts, F.M. & Abee, T. (1995) Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of *Campylobacter jejuni*. Applied and Environmental Microbiology, 61 (7), 2713-2719

Hazeleger, W.C., Wouters, J.A., Rombouts, F.M. & Abee, T. (1998) Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. Applied and Environmental Microbiology, 64 (10), 3917-3922

Heidelberg, J.F., Shahamat, M., Levin, M., Rahman, I., Stelma, G., Grim, C. & Colwell, R.R. (1997) Effect of aerosolization on culturability and viability of Gram-negative bacteria. Applied and Environmental Microbiology, 63 (9), 3585-3588

Henningson, E.W., Krocova, Z., Sandstrom, G. & Forsman, M. (1998) Flow cytometric assessment of the survival ratio of *Francisella tularensis* in aerobiological samples. FEMS Microbiology Ecology, 25 (3), 241-249

Hiraga, S., Niki, H., Ogura, T., Ichinose, C., Mori, H., Ezaki, B. & Jaffé, T. (1989) Chromosome partitioning in *Escherichia coli*: novel mutants producing anucleate cells. Journal of Bacteriology, 171, 1496-1505

Hobbie, J.E., Daley, R.J. & Jasper, S. (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Applied and Environmental Microbiology, 33 (5), 1225-1228

Hoffman, P.S., Kreig, N.R. & Smibert, R.M. (1979a) Studies of the microaerophilic nature of *Campylobacter fetus* subsp *jejuni*. I. Physiological aspects of enhanced aerotolerance. Canadian Journal of Microbiology, 25, 1-7

Hoffman, P.S., Kreig, N.R. & Smibert, R.M. (1979b) The microaerophilic nature of *Campylobacter fetus* subsp *jejuni*. II. Role of exogenous superoxide anions and hydrogen peroxide. Canadian Journal of Microbiology, 25, 8-16

Höller, C., Witthuhn, D. & Janzen-Blunck, B. (1998) Effect of low temperatures on growth, structure, and metabolism of *Campylobacter coli* SP10. Applied and Environmental Microbiology, 64 (2), 581-587

Hughes, R.A.C. & Rees, J.H. (1997) Clinical and epidemiologic features of Guillain-Barré syndrome. The Journal of Infectious Diseases, 176 (suppl 2), S92-S98

Huisman, G.W., Siegele, D.H., Zambrano, M.M. & Kolter, R. (1996) Morphological and physiological changes during stationary phase. Chapter 106, p1672-1683. *In. Escherichia coli* and *Salmonella*: cellular and molecular biology, Vol. II, Neidhardt, F.C (ed), ASM Press, Washington D.C

Humphrey, T.J. & Cruickshank, J.G. (1985) Antibiotic and deoxycholate resistance in *Campylobacter jejuni* following freezing or heating. Journal of Applied Bacteriology, 59, 65-71

Humphrey, T.J. & Hart, R.J.C. (1988) Campylobacter and salmonella contamination of unpasteurized cows' milk on sale to the public. Journal of Applied Bacteriology, 65, 463-467

Hwang, C-A. & Beuchat, L.R. (1995) Efficacy of selected chemicals for killing pathogenic and spoilage microorganisms on chicken skin. Journal of Food Protection, 58 (1), 19-23

Iuchi, S. & Weiner, L. (1996) Cellular and molecular physiology of *Escherichia coli* in the adaptation to aerobic environments. Journal of Biochemistry, 120, 1055-1063

Jacob, J., Martin, W. & Höller, C. (1993) Characterisation of viable-but nonculturable stage of *C. coli*, characterised with respect to electron microscopic findings, whole cell protein and lipooligosaccharide (LOS) patterns. Zentralblatt für Mikrobiologie, 148, 3-10

Jacobsen, C.N., Rasmussen, J. & Jakobsen, M. (1997) Viability staining and flow cytometric detection of *Listeria monocytogenes*. Journal of Microbiological Methods, 28, 35-43

Jeffrey, W.H. & Mitchell, D.L. (1997) Mechanisms of UV-induced DNA damage and response in marine microorganisms. Photochemistry and Photobiology, 65 (2), 260-263

Jepras, R.I., Carter, J., Pearson, S.C., Paul, F.E. & Wilkinson, M.J. (1995) Development of a robust flow cytometric assay for determining numbers of viable bacteria. Applied and Environmental Microbiology, 61 (7), 2696-2701

Jones, D.M., Sutcliffe, E.M. & Curry, A. (1991a) Recovery of viable but non-culturable *Campylobacter jejuni*. Journal of General Microbiology, 137, 2477-2484

Jones, D.M., Curry, A. & Sutcliffe, E.M. (1991b) Coccal forms of campylobacters and *Helicobacter* are generated differently. Microbial Ecology in Health and Disease, 4, S76

Jones, D.M., Sutcliffe, E.M., Rios, R., Fox, A.J. & Curry, A. (1993) *Campylobacter jejuni* adapts to aerobic metabolism in the environment. Journal of Medical Microbiology, 38, 145-150

Jones, I.G. & Roworth, M. (1996) An outbreak of *Escherichia coli* O157 and campylobacteriosis associated with contamination of a drinking water supply. Public Health, 110, 277-282

Jones, K., Betaieb, M. & Telford, D.R. (1990a) Seasonal variation of thermophilic campylobacters in sewage sludge. Journal of Applied Bacteriology, 69, 185-189

Jones, K., Betaieb, M. & Telford, D.R. (1990b) Correlation between environmental monitoring of thermophilic *Campylobacter* infection in the community. Journal of Applied Bacteriology

Jones, K., Betaieb, M. & Telford, D.R. (1990c) Thermophilic campylobacters in surface waters around Lancaster, UK: Negative correlation with *Campylobacter* infections in the community. Journal of Applied Bacteriology

Jones, K., Howard, S. & Wallace, J.S. (1999) Intermittent shedding of thermophilic campylobacters by sheep at pasture. Journal of Applied Microbiology, 86, 531-536

Jones, P.G., Krah, R., Tafuri, S.R. & Wolffe, A.P. (1992) DNA gyrase, CS7.4, and the cold shock response in *Escherichia coli*. Journal of Bacteriology, 174 (18), 5798-5802

Jones, P.G. & Inouye, M. (1994) The cold-shock response - a hot topic. Molecular Microbiology, 11, 811-818

Jordan, K.N., Oxford, L. & O'Byrne, C.P. (1999) Survival of low-pH stress by *Escherichia coli* O157:H7: correlation between alterations in the cell envelope and increased acid tolerance. Applied and Environmental Microbiology, 65 (7), 3048-3055

Joux, F., Lebaron, P. & Trousellier, M. (1997a) Changes in cellular states of the marine bacterium *Deleya aquamarina* under starvation conditions. Applied and Environmental Microbiology, 63 (7), 2686-2694

Joux, F., Lebaron, P. & Trousellier, M. (1997b) Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. FEMS Microbiology Ecology, 22, 65-76

Kapperud, G. & Rosef, O. (1983) Avian wildlife reservoir of *Campylobacter fetus* subsp. *jejuni*, *Yersinia* spp, and *Salmonella* spp in Norway. Applied and Environmental Microbiology, 45 (2), 375-380

Kaprelyants, A.S. & Kell, D.B. (1992) Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. Journal of Applied Bacteriology, 72, 410-422

Kaprelyants, A.S., Mukamolova, G.V. & Kell, D.B. (1994) Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell-free spent culture medium at high dilution. FEMS Microbiology Letters, 115 (2-3), 347-352

Kapuscinski, J. & Szer, W. (1979) Characterisation of interaction between DNA and 4',6-diamidino-2-phenylindole by optical spectroscopy. Nucleic Acids Research, 6, 3519-3534

Karmali, M.A., Allen, A.K. & Fleming, P.C. (1981) Differentiation of catalase-positive campylobacters with special reference to morphology. International Journal of Systemic Bacteriology, 31 (1), 64-71

Karmali, M.A., Williams, A., Fleming, P.C., Krishnan, C. & Wood, M.M. (1984) Use of an ammonium electrode to study bacterial deamination of amino acids with special reference to D-asparaginase breakdown by Campylobacters.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R. & Barer, M.R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie van Leeuwenhoek, 73, 169-187

Ketley, J.M. (1997) Pathogenesis of enteric infection by *Campylobacter*. Microbiology, 143, 5-21

Kjelleberg, S., Albertson, N., Flärdh, K., Holmqvist, L., Jouper-Jaan, Å., Marouga, R., Östling, J., Svenblad, B. & Weichart, D. (1993) How do non-differentiating bacteria adapt to starvation? Antonie van Leeuwenhoek, 63, 333-341

Koechlein, D.J. & Kreig, N.R. (1998) Viable but nonculturable coccoid forms of *Prolinoborus fasciculus* (*Aquaspirillum fasciculus*). Canadian Journal of Microbiology, 44, 910-912

Koenraad, P.M.J.F., Rombouts, F.M. & Notermans, S.H.W. (1997) Epidemiological aspects of thermophilic *Campylobacter* in water-related environments: a review. Water Environment Research, 69, 52-63

Kogure, K., Simidu, U. & Taga, N. (1979) A tentative direct microscopic method for counting living marine bacteria. Canadian Journal of Microbiology, 25, 415-420

Koike, Y. & Shimazaki, Y. (1982) Studies on the coccoid form of *Campylobacter* species: fluctuation in cellular morphology. Bulletin of Nippon Veterinary and Zootechnical College, 31, 136-142

Kolter, R., Siegele, D.A. & Tormo, A. (1993) The stationary phase of the bacterial life cycle. Annual Reviews in Microbiology, 47, 855-874

Konkel, M.E. & Cieplak, W., Jr. (1992) Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalisation. Infection and Immunity, 60 (11), 4945-4949

Konkel, M.E., Marconi, R.T., Mead, D.J. & Cieplak Jr., W. (1994) Cloning and expression of the *hup* gene encoding a histone-like protein of *Campylobacter jejuni*. Gene, 146, 83-86

Konkel, M.E., Kim, B.J., Klena, J.D., Young, C.R. & Ziprin, R. (1998) Characterisation of the thermal stress response of *Campylobacter jejuni*. Infection and Immunity, 66 (8), 3666-3672

Korhonen, L.K. & Martikainen, P.J. (1991a) Survival of *Escherichia coli* and *Campylobacter jejuni* in untreated and filtered lake water. Journal of Applied Bacteriology, 71, 379-382

Korhonen, L.K. & Martikainen, P.J. (1991b) Comparison of the survival of *Campylobacter jejuni* and *Campylobacter coli* in culturable form in surface water. Canadian Journal of Microbiology, 37, 530-533

Korman, T.M., Varley, C.C. & Spelman, D.W. (1997) Acute hepatitis associated with *Campylobacter jejuni* bacteraemia. European Journal of Clinical Microbiology & Infectious Disease, 16 (9), 678-681

Kusters, J.G., Gerrits, M.M., van Strijp, J.A.G. & van den Broucke-Grauls, C.M.J.E. (1997) Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. Infection and Immunity, 65 (9), 3672-3679

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685

Lambert, L.A., Abshire, K., Blankenhorn, D. & Slonczewski, J.L. (1997) Proteins induced in *Escherichia coli* by benzoic acid. Journal of Bacteriology, 179, 7595-7599

Lázaro, B., Cárcamo, J., Audícana, A., Perales, I. & Fernández-Astorga, A. (1999) Viability and DNA maintenance in nonculturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. Applied and Environmental Microbiology, 65 (10), 4677-4681

Lazazzera, B.A. & Grossman, A.D. (1998) The ins and outs of peptide signalling. Trends in Microbiology, 6 (7), 288-294

Leach, S., Harvey, P. & Wait, R.C. (1997) Changes with growth rate in the membrane lipid composition and amino acid utilization by continuous cultures of *Campylobacter jejuni*. Journal of Applied Microbiology, 82 (5), 631-640

Lee, K.H. & Ruby, E.G. (1995) Symbiotic role of the viable but nonculturable state of *Vibrio fischeri* in Hawaiian coastal seawater. Applied and Environmental Microbiology, 61 (1), 278-283

Lee, I.S., Lin, J., Hall, H.K., Bearson, B. & Foster, J.W. (1995) The stationary-phase sigma factor σ^s (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. Molecular Microbiology, 17, 155-167

Leung, K.T., Cassidy, M.B., Holmes, S.B., Lee, H. & Trevors, J.T. (1995) Survival of κ-carrageenan-encapsulated and unencapsulatd *Pseudomonas aeruginosa* UG2LR cells in forest soil monitored by polymerase chain reaction and spread plating. FEMS Microbiology Ecology, 16 (1), 71-82

Li, Y., Walker, J.T., Slavik, M.F. & Wang, H. (1995) Electrical treatment of poultry chiller water to destroy *Campylobacter jejuni*. Journal of Food Protection, 58 (12), 1330-1334

Li, C., Wu, P.Y. & Hsieh, M. (1997) Growth phase dependent transcriptional regulation of the *pcm* and *sur*E genes required for stationary phase survival of *Escherichia coli*. Microbiology, 143 (11), 3513-3520

Lichtman, S.N. (1996) Role of endogenous enteric organisms in the reactivation of arthritis. Molecular Medicine Today, 20, 385--391

Lin, J., Lee, I.S., Frey, J., Slonczewski, J.L. & Foster, J.W. (1995) Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*. Journal of Bacteriology, 177, 4097-4014

López-Amorós, R., Comens, J. & Vives-Rego, J. (1995) Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation-survival in seawater using rhodamine 123, propidium iodide, and oxonol. Applied and Environmental Microbiology, 61 (7), 2521-2526

Lowrie, D.B., Pearce, J.H. & Kennedy, J.F. (1974) The effect of osmotic variation upon the growth of *Vibrio fetus*. Journal of General Microbiology, 80, 541-548

Maeda, H. & Ishida, N. (1967) Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. The Journal of Biochemistry, 62 (2), 276-278

Manahan, S.H. & Steck, T.R. (1997) The viable but nonculturable state in *Agrobacterium tumefaciens* and *Rhizobium meliloti*. FEMS Microbiology Ecology, 22, 29-37

Marais, A., Mendz, G.L., Hazell, S.L. & Megraud, F. (1999) Metabolism and genetics of *Helicobacter pylori*: The genome era. Microbiology and Molecular Biology Reviews, 63 (3), 642-677

Margariños, B., Romalde, J.L., Cid, A. & Toranzo, A.E. (1997) Viability of starved *Pasteurella piscicida* in sea water monitored by flow cytometry and the effect of antibiotics on its resuscitation. Letters in Applied Microbiology, 24 (2), 122-126

Marsh, D.H., Kluses, E.S. & Ogg, J.E. (1969) Separation of cell types of *Vibrio fetus*. Applied Microbiology, 18 (6), 1096-1097

Mason, D.J., Lopéz-Amorós, R., Allman, R., Stark, J.M. & Lloyd, D. (1995) The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. Journal of Applied Bacteriology, 78, 309-315

Matin, A. (1992) Physiology, molecular biology and applications of the bacterial starvation response. Journal of Applied Bacteriology Symposium Supplement, 73, 49S-57S

McDougald, D., Rice, S.A., Weichart, D. & Kjelleberg, S. (1998) Nonculturability: adaptation or debilitation? FEMS Microbiology Ecology, 25, 1-9

McKay, A.M. (1992) Viable but non-culturable forms of potentially pathogenic bacteria in water. Letters in Applied Microbiology, 14, 129-135

Medema, G.J., Schets, F.M., Van de Giessen, A.W. & Havelaar, A.H. (1992) Lack of colonisation of 1 day old chicks by viable, non-culturable *Campylobacter jejuni*. Journal of Applied Bacteriology, 72, 512-516

Merrell, B.R., Walker, R.I. & Coolbaugh, J.C. (1981) *Campylobacter fetus* ss. *jejuni*, a newly recognised enteric pathogen: morphology and intestinal colonisation. Scanning Electron Microscopy, 4, 125-131

Meynell, G.G. & Meynell, E. (1965) Quantitative aspects of microbiological experiments. Chapter 6, p147-181. *In.* Theory and practice in experimental bacteriology. Cambridge University Press

Michel, V., Lehoux, I., Depret, G., Anglade, P., Labadie, J. & Hebraud, M. (1997) The cold shock response of the psychrotrophic bacterium *Pseudomonas fragi* involves four low-molecular-mass nucleic acid-binding proteins. Journal of Bacteriology, 179 (23), 7331-7342

Miller, R.A. & Brittigan, B.E. (1997) Role of oxidants in microbial pathophysiology. Clinical Microbiology Reviews, 10 (1), 1-18

Minakshi, S.C.D. & Ayyagari, A. (1988) Isolation of *Campylobacter jejuni* from quails: an initial report. British Veterinary Journal, 144, 411-412

Minder, H.C., Fischer, H.M., Hennecke, H. & Narberhaus, F. (2000) Role of HrcA and CIRCE in the heat-shock regulatory network of *Bradyrhizobium japonicum*. Journal of Bacteriology, 182 (1), 14-22

Monfort, P. & Baleux, B. (1994) Effects of environmental factors present in the St. Lawrence estuary (Quebec, Canada) on experimental survival of *Salmonella salamae* as determined by flow cytometry. Canadian Journal of Microbiology, 40, 712-719

Moore, J.M. (1996) Occurrence of thermophilic Campylobacter spp. in fods and waters in Northern Ireland, p135-139. *In*, *Campylobacters*, *Helicobacters*, and Related Organisms. Newell, D.G, Ketley, J, & Feldman, R.A (eds), New York, Plenum

Moran, A.P. & Upton, M.E. (1986) A comparative study of the rod and coccoid forms of *Campylobacter jejuni* ATCC 29428. Journal of Applied Bacteriology, 60, 103-110

Moran, A.P. & Upton, M.E. (1987a) Effect of medium supplements, illumination and superoxide dismutase on the production of coccoid forms of *Campylobacter jejuni* ATCC 29428. Journal of Applied Bacteriology, 72, 512-516

Moran, A.P. & Upton, M.E. (1987b) Factors affecting production of coccoid forms *by Campylobacter jejuni* on solid media during incubation. Journal of Applied Bacteriology, 62, 527-537

Morgan, R.W., Christman, M.F., Jacobsen, F.S., Storz, G. & Ames, B.N. (1986) Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proceedings of the National Academy of Science USA, 83, 8059-8063

Morgan, J.A.W., Cranwell, P.A. & Pickup, R.W. (1991) Survival of *Aeromonas salmonicida* in lake water. Applied and Environmental Microbiology, 57 (6), 1777-1782

Morgan, J.A.W., Kuntzelmann, V., Tavernor, S., Ousley, M.A. & Winstanley, C. (1997) Survival of *Xenorhabdus nematophilus* and *Photorhabdus luminescens* in water and soil. Journal of Applied Microbiology, 83, 665-670

Mukherjee, T.K., Raghavan, A. & Chatterji, D. (1998) Shortage of nutrients in bacteria: The stringent response. Current Science, 75 (7), 684-689

Muller, T. & Seyfarth, W. (1997) Starvation and nonculturable state in plant-associated lactic acid bacteria. Microbiological Research, 152 (1), 39-43

Munro, P.M., Flatau, G.N., Clément, R.L. & Gauthier, M.J. (1994) Influence of the RpoS (KatF) Sigma factor on the maintenance of culturability of *Escherichia coli* and *Salmonella typhimurium* in seawater. Applied and environmental Microbiology, 61, 1853-1858

Munroe, D.L., Prescott, J.F. & Penner, J.L. (1983) *Campylobacter jejuni* and *Campylobacter coli* serotypes isolated from chickens, cattle and pigs. Journal of Clinical Microbiology, 18, 877-878

Nachamkin, I., Allos, B.M. & Ho, T. (1998) *Campylobacter* species and Guillain-Barré syndrome. Clinical Microbiology Reviews, 11 (3), 555-567

Nebe-Von Caron, G. & Badley, R.A. (1995) Viability assessment of bacteria in mixed populations using flow cytometry. Journal of Microscopy, 79 (1), 55-66

Nebe-Von Caron, G., Stephens, P. & Badley, A.R. (1999) Bacterial detection and differentiation by cytometry and fluorescent probes. Royal Microscopical Society Proceedings, 34 (1), 321-327

Nelson, D.R., Sadlowski, Y., Eguchi, M. & Kjelleberg, S. (1997) The starvation-stress response of *Vibrio* (*Listonella*) *anguillarum*. Microbiology, 143 (7), 2305-2312

Ng, L.K., Sherburne, R., Taylor, D.E. & Stiles, M.E. (1985) Morphological forms and viability of *Campylobacter* species studied by electron microscopy. Journal of Bacteriology, 164 (1), 338-343

Nikaido, H. & Vaara, M. (1985) Molecular basis of bacterial outer membrane permeability. Microbiological Reviews, 49 (1), 1-32

Nikaido, H. (1994) Porins and specific diffusion channels in bacterial outer membranes. Journal of Biological Chemistry, 269 (6), 3905-3908

Nilius, M., Ströhle, A., Bode, G. & Malfertheiner, P. (1993) Coccoid like forms (CLF) of *Helicobacter pylori*. Enzyme activity and antigenicity. Zentralblatt fur Bakteriologie, 280, 259-272

Nilsson, L., Oliver, J.D. & Kjelleberg, S. (1991) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Journal of Bacteriology, 173 (6), 5054-5059

Nováková, R., Sevcikova, B. & Kormanec, J. (1998) A method for the identification of promoters recognized by RNA polymerase containing a particular sigma factor: cloning of a developmentally regulated promoter and corresponding gene directed by the *Streptomyces aureofaciens* sigma factor, RpoZ. Gene, 208 (1), 43-50

Nwoguh, C.E., Harwood, C.R. & Barer, M.R. (1995) Detection of induced β-galactosidase activity in individual non-culturable cells of pathogenic bacteria by quantitative cytological assay. Molecular Microbiology, 17 (3), 545-554

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. The Journal of Biological Chemistry, 250 (10), 4007-4021

Ogg, J.E. (1962) Studies on the coccoid form of ovine *Vibrio fetus* I. Cultural and serologic investigations. American Journal of Veterinary Research, 23, 354-358

Oliver, J.D. (1993) Formation of viable but nonculturable cells. *In.* Starvation in Bacteria, Kjelleberg, S (ed), Plenum Press, New York

Oliver, J.D. & Bockian, R. (1995) *In vivo* resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Applied and Environmental Microbiology, 61, 2620-2623

Östling, D., McDougald, D., Marouga, R. & Kjelleberg, S. (1997) Global analysis of physiological responses in marine bacteria. Electrophoresis, 18, 1441-1450

Pace, J.L., Chai, T.J., Rossi, H.A. & Jiang, X.P. (1997) Effect of bile salts on *Vibrio parahaemolyticus*. Applied and Environmental Microbiology, 63 (6), 2372-2377 VBNC

Pacha, R.E., Clark, G.W., Williams, E.A. & Carter, A.M. (1988) Migratory birds of central Washington as reservoirs of *Campylobacter jejuni*. Canadian Journal of Microbiology, 34, 80-82

Palmer, S.R., Gully, P.R., White, J.M., Pearson, A.D., Suckling, W.G., Jones, D.M,. Rawes, J.C.L. & Penner, J.L. (1983) Water-borne outbreak of campylobacter gastroenteritis. The Lancet, (I), 287-290

Panoff, J-M., Thammavongs, B., Guéguen, E. & Boutibonnes, P. (1998) Cold stress responses in mesophilic bacteria. Cryobiology, 36, 75-83

Palumbo, S.A. (1984) Heat injury and repair in *Campylobacter jejuni*. Applied and Environmental Microbiology, 48 (3), 477-480

Park, R.W.A., Griffiths, P.L. & Moreno, G.S. (1991) Sources and survival of campylobacters: relevance to enteritis and the food industry. Journal of Applied Bacteriology Symposium Supplement, 70, 97S-106S

Paulsen, I.T., Brown, M.H. & Skurray, R.A. (1996) Proton-dependent multidrug efflux systems. Microbiological Reviews, 60 (4), 575-608

Pead, P.J. (1979) Electron microscopy of *Campylobacter jejuni*. Journal of Medical Microbiology, 12, 383-385

Pearson, A.D., Greenwood, M., Healing, T.D., Rollins, D., Shahamat, M., Donaldson, J. & Colwell, R.R. (1993) Colonisation of broiler chickens by waterborne *Campylobacter jejuni*. Applied and Environmental Microbiology, 59 (4), 987-996

Peterson, G.L. (1977) A simplification of the protein assay method of Lowry *et al.* which is generally more applicable. Analytical Biochemistry, 83, 346-356

Phadtare, S., Alsina, J. & Inouye, M. (1999) Cold-shock response and cold-shock proteins. Current Opinion in Microbiology, 2 (2), 175-180

Pine, L., Hoffmann, P.S., Malcolm, G.B., Benson, R.F. & Franzus, M.J. (1986) Role of keto acids and reduced-oxygen-scavenging enzymes in the growth of *Legionella* species. Journal of Clinical Microbiology, 23 (1), 33-42

Pommepuy, M., Butin, M., Derrien, A., Gourmelon, M., Colwell, R.R. & Cormier, M. (1996) Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight. Applied and Environmental Microbiology, 62 (12), 4621-4626

Purdy, D. & Park, S.F. (1994) Cloning, nucleotide sequence and characterisation of a gene encoding superoxide dismutase from *Campylobacter jejuni* and *Campylobacter coli*. Microbiology, 140, 1203-1208

Qi, H.Y., Menzel, R. & TseDinh, Y.C. (1997) Regulation of *Escherichia coli top*A gene transcription: Involvement of a σ^S-dependent promoter. Journal of Molecular Biology, 267 (3), 481-489

Rahman, I., Shahamat, M., Kirchman, P.A., Russek-Cohen, E. & Colwell, R.R. (1994) Methionine uptake and cytopathogenicity of viable but nonculturable *Shigella dysenteriae* type 1. Applied and Environmental Microbiology, 60, 3573-3578

Rahman, I., Shahamat, M., Chowdhury, M.A.R. & Colwell, R.R. (1996) Potential virulence of viable but nonculturable *Shigella dysenteriae* type 1. Applied and Environmental Microbiology, 62 (1), 115-120

Ravel, J., Knight, I.T., Monahan, C.E., Hill, R.T. & Colwell, R.R. (1995) Temperature-induced recovery of *Vibrio cholerae* from the viable but nonculturable state: growth or resuscitation? Microbiology, 141, 377-383

Ray, B. (1984) Reversible freeze-injury. Chapter 8, p238-272. *In.* Repairable lesions in microorganisms. Hurst, A & Nasim, A (eds). Academic Press, London

Ray, B. & Johnson, C. (1984) Survival and growth of freeze-stressed *Campylobacter jejuni* cells in selective media. Journal of food Safety, 6 (3), 183-195

Reezal, A., McNeil, B. & Anderson, J.G. (1998) Effect of low-osmolarity nutrient media on growth and culturability of *Campylobacter* species. Applied and Environmental Microbiology, 64 (12), 4643-4649

Rice, S.A., Givskov, M., Steinberg, P. & Kjelleberg, S. (1999) Bacterial signals and antagonists: The interaction between bacteria and higher organisms. Journal of Molecular Microbiology and Biotechnology, 1 (1), 23-32

Rigsbee, W., Simpson, L.M. & Oliver, J.D. (1997) Detection of the viable but nonculturable state in *Escherichia coli* O157:H7. Journal of Food Safety, 16 (4), 255-262

Robinson, D.A. (1981) Infective dose of *Campylobacter jejuni* in milk. British Medical Journal, 282, 1584

Rocha, E.R., Selby, T., Coleman, J.P. & Smith, C.J. (1996) Oxidative stress response in an anaerobe, *Bacteroides fragilis*: a role for catalase in protection against hydrogen peroxide. Journal of Bacteriology, 178 (23), 6895-6903

Rodriguez, G.G., Phipps, D., Ishiguro, K. & Ridgeway, H.F. (1992) Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria. Applied and Environmental Microbiology, 58 (6), 1801-1808

Rollins, D.M., Coolbaugh, J.C., Walker, R.I. & Weiss, E. (1983) Biphasic culture system for rapid *Campylobacter* cultivation. Applied and Environmental Microbiology, 45 (1), 284-289

Rollins, D.M. & Colwell, R.R. (1986) Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Applied and Environmental Microbiology, 52 (3), 531-538

Romalde J., Barja, J.L., Margariños, R. & Toranzo, A.E. (1994) Starvation-survival processes of the bacterial fish pathogen *Yersinia ruckeri*. Systemic and Applied Microbiology, 17, 161-168

Romeo, T. (1998) Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule, CsrB. Molecular Microbiology, 29 (6), 1321-1330

Rosef, O. & Kapperud, G. (1983) House-flies (*Musca domestica*) as possible vectors of *Campylobacter* subsp *jejuni*. Applied and Environmental Microbiology, 45 (2), 381-383

Roszak,D.B., Grimes, D.J. & Colwell, R.R. (1984) Viable but nonrecoverable stage of *Salmonella entertidis* in aquatic systems. Canadian Journal of Microbiology, 30, 334-338

Roszak, D.B. & Colwell, R.R. (1987a) Survival strategies of bacteria in the natural environment. Microbiological Reviews, 51 (3), 365-379

Roszak, D.B. & Colwell, R.R. (1987b) Metabolic activity of bacterial cells enumerated by direct viable count. Applied and Environmental Microbiology, 53 (12), 2889-2983

Roth, W.G., Leckie, M.P. & Dietzler, D.N. (1988) Restoration of colony-forming activity in osmotically stressed *Escherichia coli* by betaine. Applied and Environmental Microbiology, 54 (12), 3142-3146

Rothfield, L.I. & Justice, S.S. (1997) Bacterial cell division: The cycle of the ring. Cell, 88 (5), 581-584

Rowe, M.T., Dunstall, G., Kirk, R., Loughney, C.F., Cooke, J.L. & Brown, S.R. (1998) Development of an image system for the study of viable but non-culturable forms of *Campylobacter jejuni* and its use to determine their resistance to disinfectants. Food Microbiology, 15, 491-498

Saha, S.K., Saha, S. & Sanyal, S.C. (1991) Recovery of injured *Campylobacter jejuni* cells after animal passage. Applied and Environmental Microbiology, 57 (11), 3388-3389

Sahay, P., West, A.P., Birkenhead, D. & Hawkey, P.M. (1995) *Campylobacter jejuni* in the stomach. Journal of Medical Microbiology, 43 (1), 75-77

Sakuma, T., Yamada, N., Saito, H., Kakegawa, T. & Kobayashi, H. (1998) pH dependence of the function of sodium ion extrusion systems in *Escherichia coli*. Biochimica et biophysica acta - bioenergetics, 1363 (3), 231-237

Saleha, A.A., Mead, G.C. & Ibrahim, A.L. (1998) *Campylobacter jejuni* in poultry production and processing in relation to public health. World's Poultry Science Journal, 54, 49-58

Sanders, J.W., Venema, G. & Kok, J. (1999) Environmental stress responses in *Lactococcus lactis*. FEMS Microbiology Reviews, 23, 483-501

Santiwatanakul, S. & Kreig, N.R. (1999) Isolation and purification of a *Campylobacter upsaliensis* autolysin. Canadian Journal of Microbiology, 45, 23-30

Segura, A., Duque, E., Mosqueda, G., Ramos, J.L. & Junker, F. (1999) Multiple responses of Gram-negative bacteria to organic solvents. Environmental Microbiology, 1 (3), 191-198

Servis, N.A., Nichols, S. & Adams, J.C. (1995) Development of a direct viable count procedure for some Gram-positive bacteria. Letters in Applied Microbiology, 20 (4), 237-239

Setlow, P. (1992) I will survive - protecting and repairing spore DNA. Journal of Bacteriology, 174 (9), 2737-2741

Shahamat, M., Mai, U., Pazko-Kolva, C., Kessel, M. & Colwell, R.R. (1993) Use of autoradiography to assess viability of *Helicobacter pylori* in water. Applied and Environmental Microbiology, 59 (4), 1231-1235

Shibata, Y., Kasahara, Y. & Amano, K. (1991) Morphological changes of *Campylobacter jejuni* and *Campylobacter coli* under various culture conditions and the accompanied changes in the cell composition. Japanese Journal of Bacteriology, 46 (6), 921-927

Siegele, D.A. & Kolter, R. (1992) Life after log. Journal of Bacteriology, 174 (2), 345-348

Siegele, D.A., Imlay, K.R. & Imlay, J.A. (1996) The stationary phase exit defect of *cyd*C (*sur*B) mutants is due to the lack of a functional terminal cytochrome oxidase. Journal of Bacteriology, 178 (21), 6091-6096

Silver, S. & Phung, L.T. (1996) Bacterial heavy metal resistance: New surprises. Annual Review of Microbiology, 50, 753-789

Skirrow, M.B. (1990) Foodborne illness: Campylobacter. The Lancet, 336 (Oct 13th), 921-923

Slavik, M.F., Kim, J-W., Pharr, M.D., Raben, D.P., Tsai, S. & Lobsinger, C.M. (1994) Effect of trisodium phosphate on *Campylobacter* attached to post-chill chicken carcasses. Journal of Food Protection, 57 (4), 324-326

Small, P., Blankenhorn, D., Welty, D., Zinser, E. & Slonczewski, J.L. (1994) Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of rpoS and growth pH. Journal of Bacteriology, 176 (6), 1729-1737

Smibert, R.M. (1978) The genus *Campylobacter*. Annual Reviews in Microbiology, 32, 673-709

Smith, J.J., Howington, J.P. & McFeters, G.A. (1994) Survival, physiological response, and recovery of enteric bacteria exposed to a polar marine environment. Applied and Environmental Microbiology, 60 (8), 2977-2984

Smith, J.J. & McFeters, G.A. (1997) Mechanism of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in *Escherichia coli* K-12. Journal of Microbiological Methods, 29 (3), 161-175

Spohn, G. & Scariato, V. (1999) The autoregulatory HspR repressor protein governs chaperone gene transcription in *Helicobacter pylori*. Molecular Microbiology, 34 (4), 663-674

Stanley, K.N., Wiallace, J.S., Currie, J.E., Diggle, P.J. & Jones, K. (1998) Seasonal variation of thermophilic Campylobacters in lambs at slaughter. Journal of Applied Microbiology, 84 (6), 1111-1116

Steinert, M., Emödy, L., Amann, P.G. & Hacker, J. (1997) Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Applied and Environmental Microbiology, 63 (5), 2047-2053

Stephens, C.P., On, S.L.W. & Gibson, J.A. (1998) An outbreak of infectious hepatitis in commercially reared ostriches associated with *Campylobacter coli* and *Campylobacter jejuni*. Veterinary Microbiology, 61 (3), 183-190

Stern, N.J., Jones, D.M., Wesley, I.V. & Rollins, D.M. (1994) Colonisation of chicks by non-culturable *Campylobacter spp.* Letters in Applied Microbiology, 18, 333-336

Suzuki, S., Horikoshi, Y. & Takama, K. (1994) Changes in cell morphology and polyamine composition during growth of *Campylobacter jejuni*. World Journal of Microbiology and Biotechnology, 10, 352-353

Swift, S., Williams, P. & Stewart, G.S.A.B. (1999) *N*-acylhomoserine lactones and quorum sensing in proteobacteria, p291-313 *In*. Cell-Cell Signalling In Bacteria, Dunny, G.M & Winans, S.C (eds), American Society for Microbiology, Washington D.C

Takata, T., Wai, S.N., Takade, A., Sawae, Y., Ono, J. & Amako, K. (1995) The purification of a GroEL-like stress protein from aerobically adapted *Campylobacter jejuni*. Microbiology and Immunology, 39 (9), 639-645

Talsma, E., Goettsch, W.G., Nieste, H.L.J., Schrijnemakers, P.M. & Sprenger, M.J.W. (1999) Resistance in *Campylobacter* species: Increases resistance to fluoroquinolones and seasonal variation. Clinical Infectious Diseases, 29 (4), 845-848

Tanabe, H., Goldstein, J., Yang, M. & Inouye, M. (1992) Identification of the promoter region of the *Escherichia coli* major cold shock gene, *csp*A. Journal of Bacteriology, 174 (12), 3867-3873

Tenover, F.C. & Fennell, C.L. (1991) Chapter 189: The genera Campylobacter and Helicobacter, p348-3511, in The Prokaryotes: A handbook on the biology of bacteria, Volume IV, Balows, A, Trüper, H.G, Dworkin, M, Harder, W, Schleifer, K-H (eds), Springer-Verlag

Terzieva, S.I. & McFeters, G.A. (1991) Survival and injury of *Escherichia coli*, *Campylobacter jejuni*, and *Yersinia enterocolitica* in stream water. Canadian Journal of Microbiology, 37, 785-790

The National Advisory Committee on Microbiological Criteria for Foods. (1994) *Campylobacter jejuni/coli*. Journal of Food Protection, 57, 1101-1121

Thies, F.L., Weishaupt, A., Karch, H., Hartung, H-P. & Giegerich, G. (1999a) Cloning, sequencing and molecular analysis of the *Campylobacter jejuni gro*ESL bicistronic operon. Microbiology, 145, 89-98

Theis, F.L., Karch, H., Hartung, H-P. & Giegerich, G. (1999b) The ClpB protein from *Campylobacter jejuni*: molecular characterisation of the encoding gene and antigenicity of the recombinant protein. Gene, 230, 61-67

Theis, F.L., Karch, H., Hartung, H-P. & Giegerich, G. (1999c) Cloning and expression of the *dna*K gene of *Campylobacter jejuni* and antigenicity of heat shock protein 70. Infection and Immunity, 67, 1194-1200

Tholozan, J.L., Cappelier, J.M., Tissier, J.P., Delattre, G. & Federghi, M. (1999) Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. Applied and Environmental Microbiology, 65 (3), 1110-1116

Thom, S.M., Horobin, R.W., Seidler, E. & Barer, M.R. (1993) Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of microbial viability and activity. Journal of Applied Bacteriology, 74, 433-443

Thomas, C., Gibson, H., Hill, D.J. & Mabey, M. (1999a) Campylobacter epidemiology: an aquatic perspective. Society for Applied Microbiology Symposium, 28, 168S-177S

Thomas, C., Hill, D.J. & Mabey, M. (1999b) Evaluation of the effect of temperature and nutrients on the survival of *Campylobacter* spp. in water microcosms. Journal of Applied Microbiology, 86 (6), 1024-1032

Thomas, C., Hill, D.J. & Mabey, M. (1999c) Morphological changes of synchronised *Campylobacter jejuni* populations during growth in single phase liquid culture. Letters in Applied Microbiology, 28 (3), 194-198

Thrane, C., Olsson, S., Nielsen, T.H. & Sorensen, J. (1998) Vital fluorescent stains for detection of stress in *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamde from *Pseudomonas fluorescens* DR54. FEMS Microbiology Ecology, 30 (1), 11-23

Torrella, F. & Morita, R.Y. (1981) Ultramicrocolony formation by heterotrophic bacteria in seawater. Applied and Environmental Microbiology, 41 (2), 518-527

Tötemeyer, S., Barrett-Bee, T.C. & Booth, I.R. (1996) Generation of a transient non-culturable state in *Pseudomonas putida* during detoxification of *N*-ethylmaleimide. Microbiology, 142, 2857-2862

Troussellier, M., Bonnefort, J-L., Courties, C., Derrien, A., Dupray, E., Gauthier, M., Gourmelon, M., Joux, F., Lebaron, P., Martin, Y. & Pommepuy, M. (1998) Responses of enteric bacteria to environmental stresses in seawater. Oceanologica Acta, 21 (6), 965-981

Ullrich, S., Karrasch, B., Hoppe, H-G., Jeskulke, K. & Mehrens, M. (1996) Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride. Applied and Environmental Microbiology, 62 (12), 4587-4593

Umunabuicke, A.C. & Irokanulo, E.A. (1986) Isolation of *Campylobacter jejuni* from Oriental and American cockroaches caught in kitchens and poultry houses in Vom, Nigeria. International Journal of Zoonoses, 13, 180-186

van de Giessen, A.W., Heuvelman, C.J., Abee, T. & Hazeleger, W.C. (1996) Experimental studies on the infectivity of non-culturable forms of *Campylobacter* spp in chicks and mice. Epidemiology and Infection, 117, 463-470

van Overbeek, L.S., Eberl, L., Givskov, M., Molin, S. & van Elsas, J.D. (1995) Survival of, and induced stress resistance in, carbon-starved *Pseudomonas fluorescens* cells residing in soil. Applied and Environmental Microbiology, 61 (12), 4202-4208

van Vliet, A.H.M., Wooldridge, K.G. & Ketley, J.M. (1998) Iron-responsive gene regulation in a *Campylobacter jejuni fur* mutant. Journal of Bacteriology, 180 (20), 5291-5298

van Vliet, A.H.M., Baillon, M-L.A., Penn, C.W. & Ketley, J.M. (1999) *Campylobacter jejuni* contains two Fur homologs: Characterisation of iron-responsive regulation of peroxide stress defense genes by the PerR repressor. Journal of Bacteriology, 181 (20), 6371-6376

Votyakova, T.V., Kaprelyants, A.S. & Kell, D.B. (1994) Influence of viable cells on the resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase: the population effect. Applied and Environmental Microbiology, 60, 3284-3291

Wai, S.N., Moriya, T., Kondo, K., Misumi, H. & Amako, K. (1996) Resuscitation of *Vibrio cholerae* 01 strain TsI-4 from a viable but-nonculturable state by heat-shock. FEMS Microbiology Letters, 136 (2), 187-191

Walkup, L.K.B. & Kogoma, T. (1989) *Escherichia coli* proteins inducible by oxidative stress mediated by the superoxide radical. Journal of Bacteriology, 171 (2), 1476-1484

Wallace, J.S., Stanley, K.N., Currie, J.E., Diggle, P.J. & Jones, K. (1997) Seasonality of thermophilic *Campylobacter* populations in chickens. Journal of Applied Microbiology, 82, 219-224

Wallace, J.S., Stanley, K.N. & Jones, K. (1998) The colonisation of turkeys by thermophilic campylobacters. Journal of Applied Microbiology, 85, 224-230

Wallis, M.R. (1994) The pathogenesis *of Campylobacter jejuni*. British Journal of Biomedical Science, 51, 57-64

Wang, W-L., L., Powers, B.W., Leuchtefeld, N.W. & Blaser, M.J. (1983) Effects of disinfectants on *Campylobacter jejuni*. Applied and Environmental Microbiology, 45 (4), 1202-1205

Wang, X., Sturegård, T., Rupar, R., Nilsson, H-O., Aleljung, P.A., Carlén, B., Willén, V. & Wadström, T. (1997) Infection of BALB/c A mice by spiral and coccoid forms of *Helicobacter pylori*. Journal of Medical Microbiology, 46, 657-663

Wang, N., Yamanaka, K. & Inouye, M. (1999) CspI, the ninth member of the CspA family of *Escherichia coli*, is induced upon cold shock. Journal of Bacteriology, 181 (5), 1603-1609

Wassenaar, T.M., Geilhausen, B. & Newell, D.G. (1998) Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. Applied and Environmental Microbiology, 64 (5), 1816-1821

Waterman, S.R. & Small, P.L.C. (1998) Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. Applied and Environmental Microbiology, 64 (10), 3882-3886

Weichart, D., Oliver, J.D. & Kjelleberg, S. (1992) Low temperature induced nonculturability and killing of *Vibrio vulnificus*. FEMS Microbiology Letters, 100 (1-3), 205-210

Weichart, D. & Kjelleberg, S. (1996) Stress resistance and recovery potential of culturable and viable but nonculturable cells of *Vibrio vulnificus*. Microbiology, 142, 845-853

Weichart, D., McDougald, D., Jacobs, D. & Kjelleberg, S. (1997) *In situ* analysis of nucleic acids in cold-induced nonculturable *Vibrio vulnificus*. Applied and Environmental Microbiology, 63 (7), 2754-2758

Weijtens, M.J.B.M., van der Plas, J., Bijker, P.G.H., Urlings, H.A.P., Koster, D., van Logtestijn, J.G. & Huis in't Veld, J.H.J. (1997) The transmission of campylobacter in piggeries; an epidemiological study. Journal of Applied Microbiology, 83, 693-698

Whitesides, M.D. & Oliver, J.D. (1997) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Applied and Environmental Microbiology, 63 (3), 1002-1005

Wilson, M. & Lindow, S.E. (1992) Relationship of total viable and culturable cells in epiphytic populations of *Pseudomonas syringae*. Applied and Environmental Microbiology, 59 (12), 3908-3913

Wu, Y.L., Lee, L.H., Rollins, D.M. & Ching, W.M. (1994) Heat shock- and alkaline pH-induced proteins of *Campylobacter jejuni*: characterisation and immunological properties. Infection and Immunity, 62 (10), 4256-4260

Xu, H-S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J. & Colwell, R.R. (1982) Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microbial Ecology, 8, 313-323

Yamanaka, K., Fang, L. & Inouye, M. (1998) The CspA family in *Escherichia coli*: multiple gene duplication for stress adaptation. Molecular Microbiology, 27 (2), 247-255

Yamanaka, K. (1999) Cold shock response in *Escherichia coli*. Journal of Molecular Microbiology and Biotechnology, 1 (2), 193-202

Yamamoto, H., Hashimoto, Y. & Ezaki, T. (1996) Study of nonculturable *Legionella pneumophila* cells during multiple-nutrient starvation. FEMS Microbiology Ecology, 20, 149-154

Yamashino, T., Kakeda, M., Ueguchi, C. & Mizuno, T. (1994) An analog of the DnaJ molecular chaperone whose expression is controlled by a σ^S during the stationary phase and phosphate starvation in *Escherichia coli*. Molecular Microbiology, 13 (3), 475-483

Yura, T. & Nakahigashi, K. (1999) Regulation of the heat-shock response. Current Opinion in Microbiology, 2 (2), 153-158

Zimmerman, R., Iturriaga, R. & Becker-Birck, J. (1978) Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Applied and Environmental Microbiology, 36 (6), 926-935